CHARACTERIZATION OF NEUTRALIZING ANTIBODY EPITOPES ON HIV-1 SUBTYPE C ENVELOPE GLYCOPROTEINS TO SUPPORT VACCINE DESIGN

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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ABSTRACT

Since its discovery as the etiological agent of AIDS in 1983, HIV-1 has been the focus of unrelenting research into an effective vaccine to control viral infection. Neutralizing antibodies constitute a correlate of immune protection for most available vaccines, but the induction of these antibodies against HIV-1 has become a major challenge. The HIV-1 envelope glycoprotein has evolved to evade neutralizing antibodies in an extraordinary way, yet a vaccine that can stimulate such antibodies remains the best hope to provide sterilizing immunity. The existence of a group of monoclonal antibodies, such as IgG1b12, 2G12, 2F5 and 4E10, capable of neutralizing a broad range of primary isolates signals vulnerable areas on the envelope glycoprotein. Furthermore, passive transfer of these antibodies can completely protect against viral challenge in animal models. The epitopes recognized by these antibodies are being intensely pursued as vaccine targets, in the hope of inducing such specificities. This thesis encompasses a series of studies on characterizing the epitopes recognized by these broadly cross-reactive monoclonal antibodies in the context of subtype C viruses. HIV-1 subtype C is responsible for the vast majority of infections worldwide, however, until recently, little research has been done on these viruses in contrast to the well characterized subtype B strains. Chapter Two describes the characterization of paediatric subtype C viruses for their sensitivity to IgG1b12, 2G12, 2F5 and 4E10. This study was done because of a planned clinical trial of some of these antibodies as post-exposure prophylaxis to prevent mother-to-child HIV-1 subtype C transmission. Only the MAb 4E10 was able to neutralize all the viruses tested, while IgG1b12 was only partially effective. 2F5 and 2G12 did not neutralize any of the viruses. The conclusion was that only 4E10 and IgG1b12 would be suitable for use as prophylactic agents in a population where HIV-1 subtype C is prevalent. Given that subtype C viruses were found to be largely insensitive to 2G12 neutralization, the commonly absent glycan at

position 295 was introduced into envelope glycoproteins from this clade. The work presented in Chapter Three explores the requirements of the 2G12 epitope on the envelopes of subtype C viruses. However, this antibody binding site was not readily reconstituted, suggesting structural differences from other HIV-1 subtypes in which the 2G12 epitope is naturally expressed. Chapter Four describes the study of 4E10 resistant virus quasispecies isolated from a seven year old perinatally HIV-1 infected child, in whom anti-MPER antibodies were found. Determinants of 4E10 neutralization were mapped to the epitope of this antibody in the MPER, as well as to the cytoplasmic tail, in particular, to four amino acids in the LLP-2 region. The role of neutralizing antibodies in natural HIV-1 subtype C infection was examined in Chapter Five by following the development of autologous and heterologous neutralizing antibodies in 14 patients during the first year of infection. Potent but relatively strain-specific neutralizing antibody responses were detected within 3-12 months of infection. The magnitude of the responses was associated with shorter V1-to-V5 envelope length and fewer glycosylation sites, in particular in the V1-V2 region. Furthermore, anti-MPER and anti-CD4i neutralizing antibodies were detected in some individuals; however, they were not associated with neutralization breadth. Finally, in Chapter Six these results are analyzed collectively, in the context of the latest findings in the field, and suggestions for further research are discussed.

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PMID: 16834457
(Chapter Two)

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Susceptibility of subtype C viruses to neutralizing monoclonal antibodies raised against subtype B
South African AIDS Conference 2005, Durban, South Africa (Oral)

Gray ES, Montefiori DC, Morris L.
Susceptibility of subtype C viruses to neutralizing monoclonal antibodies raised against subtype B
AIDS Vaccine 2005, Montreal, Canada (Poster)

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Restoration of glycan 295 in subtype C viruses renders partial sensitivity to 2G12 neutralization
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Characterization of naturally occurring 4E10 resistant viruses in a subtype C HIV-1 infected child
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Gray ES, Taylor N, Moore PL, Choge IA, Cave E, Puren A, Shaw GM, Morris L.
HIV-1 subtype C infected plasma samples with broad specificity contain anti-MPER antibodies
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I dedicate this work

To the wonderful beings from whom I came, Zoya and Suleiman,

To the special creature that came from me, Anthony, and

To Brian with whom I am.

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ABREVIATIONS

μg	microgram
AIDS	Acquired Immunodeficiency Syndrome
CAPRISA	Centre for the AIDS Program of Research in South Africa
CCR1, 2b, 3, 5, 8	chemokine (C-C motif) receptor 1, 2b, 3, 5, 8
CD4bs	CD4 binding site
CD4, CD8	cluster differentiation 4, 8
CD4i	CD4 induced
CDR	complementarity determining region
CT	cytoplasmic tail
CTL	cytotoxic T-lymphocyte
CXCR4, CXCR6	chemokine (C-X-C motif) receptor 4, 6
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin
DEAE-dextran	diethylaminoethyl-dextran
D-MEM	Dulbecco's Modified Eagle's Media
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunoabsorbent assay
ENF	enfuvirtide
env	envelope gene
Env	envelope glycoprotein
FACS	fluorescence-activated cell sorting
Gag	group associated antigen protein
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GMT	geometrical mean titer
gp120, gp41	glycoprotein 120kda, 41kda
GPR1, GPR15	G-protein receptor-1, -15
HAART	highly active antiretroviral therapy
HIV-1, HIV-2	Human Immunodeficiency Virus type-1, -2
HR-1, HR-2	first, second heptad repeat region
HTLV-1	Human T-cell Lymphotropic Virus
i.e.	*id est*, that is
IAVI	International AIDS Vaccine Initiative
IC_{50}	50% inhibitory concentration
ID_{50}	50% inhibitory dilution
IgG	immunoglobulin
LLP-1, LLP-2, LLP-3	lentivirus lytic peptide -1, -2, -3
LTR	long terminal repeat
MAb	monoclonal antibody
mg	miligram
MPER	membrane proximal external region

MTCT	Mother-to-child transmission
MuLV	Murine Leukaemia Virus
NAb	neutralizing antibodies
nMAbs	neutralizing monoclonal antibodies
p24	gag protein 24kda
PBMCs	peripherals blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNGS	potential N-linked glycosylation site
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SAAVI	South African AIDS Vaccine Initiative
sCD4	soluble CD4
SIV	Simian Immunodeficiency Virus
T-20	enfurvitide
TBS	tris buffered saline
$TCID_{50}$	50% tissue culture infectious doses
TCLA	T-cell line adapted
UNAIDS	United Nations programme on HIV/AIDS
V1, V2, V3, V4, V5	variable regions 1-5
VLPs	virus like particles

AMINO ACID ABREVIATIONS

Amino Acid	**Three-Letter code**	**One-Letter code**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Human Immunodeficiency Virus type 1 (HIV-1) has infected more than 65 million people worldwide since 1983 when it was first described as the cause of the Acquired Immune Deficiency Syndrome (AIDS). In 2007, more than 33 million people were estimated to be living with HIV-1, 2.5 million became newly infected and around 2.1 million lost their lives to AIDS (UNAIDS 2007, www.unaids.org). Although antiretroviral therapies are highly effective in treating HIV infections, only a vaccine constitutes a practical and cost-effective intervention to control the spread of the HIV/AIDS pandemic.

A major challenge for the development of an HIV vaccine has been to determine which immune responses should be elicited for protection. Currently licensed anti-viral vaccines are mostly effective against acute viral infections, conferring protection mainly through neutralizing antibodies. However, cell mediated immune responses are crucial in the control of established chronic virus infections such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Herpes simplex virus (HSV) (Pantaleo and Koup, 2004) for which it has been more difficult to develop effective vaccines. In the case of HIV, the correlates of immune protection have not been clearly identified. Early studies have showed that immunization with the outer envelope protein (gp120) of HIV-1 induced antibodies that inhibited viral entry. However, subsequent clinical studies demonstrated that these antibodies did not protect against HIV-1 infection (Gilbert *et al.*, 2005). This was later explained by their inability to neutralize primary HIV-1 isolates, despite having activity against neutralization sensitive T-cell line adapted strains (TCLA) that were used in the initial studies (Mascola and McNeil, 1995). These observations led many to question the relevance of this vaccine approach (Mammano *et al.*, 1995, Moore *et al.*, 1995, Schonning *et al.*, 1998). Soon after, a new generation of vaccine candidates emerged which aimed to prime cellular immunity, specifically $CD8^+$ cytotoxic T-lymphocytes (CTLs). Indeed,

virus-specific CTLs are thought to be responsible for controlling viral replication in the acute phase of HIV infection (Koup *et al.*, 1994). Furthermore, studies on simian immunodeficiency virus (SIV)-infected monkeys have shown that immunogens capable of inducing CTL responses can reduce viral set point and slow disease progression (Letvin, 2005). These observations suggested that vaccines capable of eliciting strong T-cell-mediated immune responses may be beneficial even if they do not induce sterilizing immunity. However, this type of response does not clear the virus reservoirs and resistance variants can emerge later in infection (Barouch *et al.*, 2003). The failure of a recent "test-of-concept" clinical trial of Merck's candidate HIV-vaccine, which aimed to stimulate CTL responses, has further questioned this vaccine approach (http://www3.niaid.nih.gov/news/newsreleases/2007/step_statement.htm). There is renewed interest in neutralizing antibodies, prompted mainly by several observations that passive transfer of neutralizing antibodies are able to confer sterilizing immunity in animal studies (Mascola, 2002). In addition, it has been shown that although effector T cells can limit viral replication, they are not able to assist humoral immunity to prevent the establishment of initial infection (Mascola *et al.*, 2003). As such, an enormous effort is currently being invested in the "intelligent" design of immunogens capable of inducing broadly cross-reactive neutralizing antibodies against HIV-1 primary isolates. Such an endeavor requires not only an in-depth understanding of the viral envelope glycoprotein structure and function, but also the role of neutralizing antibodies in natural HIV-1 infection.

1.2 HIV ENVELOPE GLYCOPROTEIN

The main targets of the anti-HIV neutralizing antibodies are the glycoprotein spikes on the virus envelope membrane. This glycoprotein complex interacts with the CD4 and coreceptor molecules present on the surface of target cell initiating the viral entry process. The functional envelope spike consists of a trimer of heterodimers formed by two

glycoproteins, gp120 (the exterior envelope glycoprotein) and gp41 (the transmembrane glycoprotein). Three gp120 molecules interact non-covalently with three gp41 units forming an oligomer, where the trimeric structure is maintained by the interactions between the gp41 domains.

In infected cells, the envelope glycoprotein is synthesized as a single polypeptide of approximately 845 to 870 amino acids in the rough endoplasmic reticulum (Allan *et al.*, 1985). The extensive addition of N-linked high-mannose sugar chains forms the gp160 glycoproteins, which assemble into trimers (Earl *et al.*, 1990). These oligomers are then transported to the Golgi apparatus, where accessible glycans are trimmed and modified to complex-oligosacharides. In the trans-Golgi, cellular proteases cleave the gp160 molecule into gp120 and gp41 (Decroly *et al.*, 1997, Hallenberger *et al.*, 1997). The mature spikes are then transported to the cell membrane, in particular to the detergent-insoluble membrane domains, known as lipid rafts (Rousso *et al.*, 2000), where the virus assembly take place and the envelope spikes are incorporated into the budding virions.

1.2.1 The gp120 molecule

The amino acid sequence of gp120 consists of five relatively conserved regions (C1-C5) interposed with five variable regions (V1-V5) which, with the exception of V5, are bracketed by cysteines forming disulfide bonds (Leonard *et al.*, 1990) (Figure 1.1). Gp120 is a highly glycosylated protein with half of its mass being N-linked glycans (Lasky *et al.*, 1986), far more glycosylated than the surface proteins from other retroviruses of similar size such as HTLV-1 and MuLV (Polonoff *et al.*, 1982). Two types of N-linked glycosylations are found on the surface of gp120, mannose-rich and complex glycans. Structural modelling suggests that the high mannose glycans are clustered on one side of the surface while the complex glycans are localized within a distinct region of the gp120 (Zhu *et al.*, 2000).

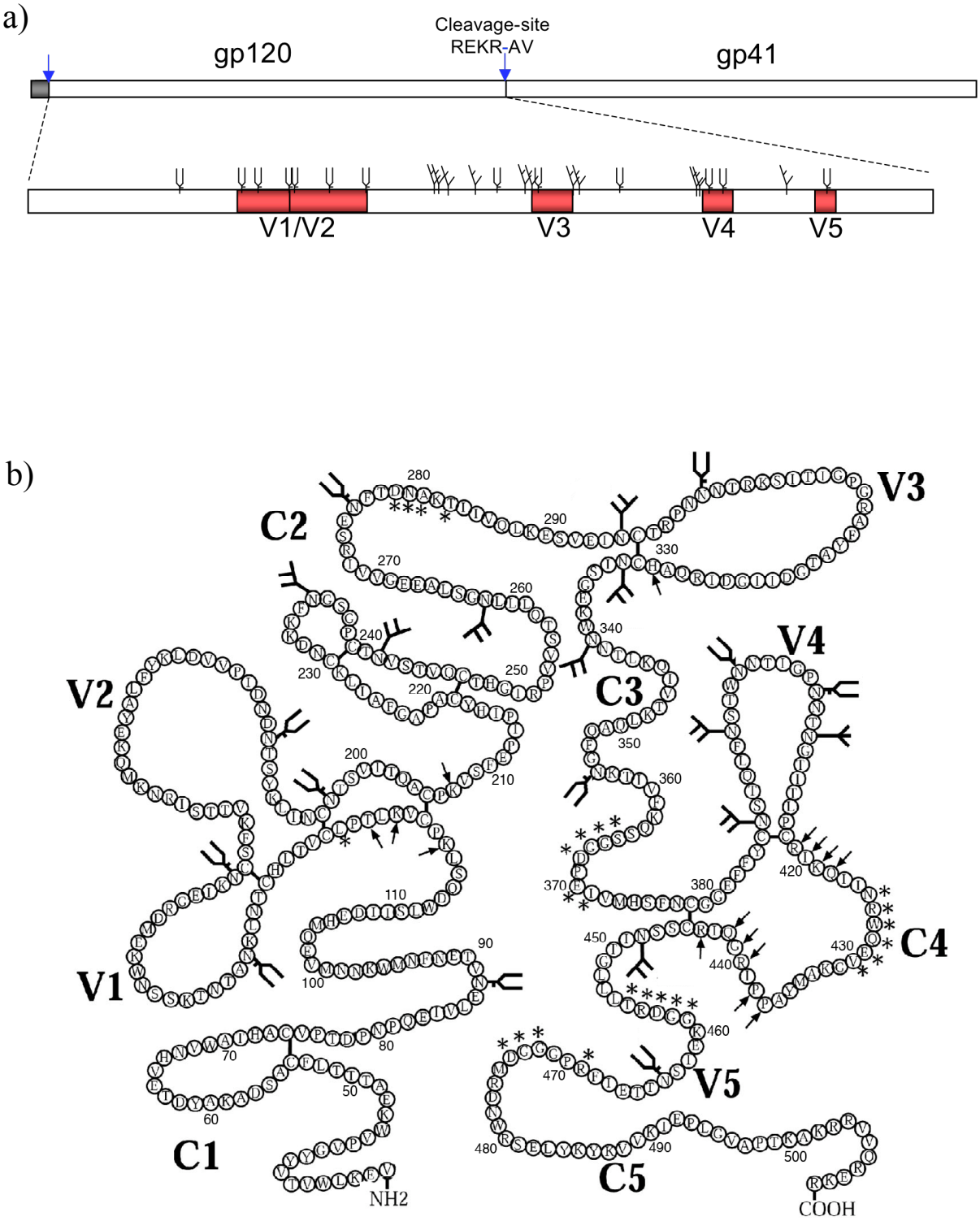


Figure 1.1 Organization of gp120 in linear and two-dimensional diagrams. (a) Schematic representation of the envelope glycoprotein precursor. Digestion by cellular proteases, at the indicated cleavage sites (vertical arrows), releases the signal peptide, the gp120 and the gp41 molecules. The locations of the variable loops (V1-V5) in gp120 are indicated in red. (b) Gp120 two-dimensional diagram adapted from McCaffrey, et al., 2004. The small numbers indicate the amino acid positions in the HxB2 sequence. Arrows indicate the positions involved in the co-receptor binding site and asterisks indicate the residues involved in CD4 binding. In both diagrams the N-linked glycosylation sites are represented by the oligosaccharides structures expected in those positions: U-shaped branches for the complex glycans and tri-branched for the mannose rich glycans.

1.2.1.1 Structural domains of gp120

Full-length gp120 has eluded structural analysis due to its lack of stability. To obtain crystal structures, HIV-1 and SIV gp120s have been deglycosylated and the N- and C-terminals, V1/V2 and V3 regions truncated, to generate what is commonly referred to as the "gp120 core". The first gp120 structure was obtained using the gp120 core of an HIV-1 virus stabilized with the D1D2 fragment of CD4 and a CD4 induced epitope-binding antibody (17b) (Kwong *et al.*, 1998). Based on this structure, gp120 is organized into three regions: the inner domain, the outer domain and the bridging sheet (Figure 1.2b). The inner domain is formed mainly by the C1 and C5 regions and is largely devoid of glycans. It has long been suggested that this domain constitutes the major contact interface with the gp41 trans-membrane unit (Helseth *et al.*, 1991, Moore *et al.*, 1994). The outer domain is heavily glycosylated and modelling of the envelope oligomer suggests that these glycans cover the solvent-exposed part of the spike, protecting it from antibody recognition. In between the outer and inner domains is the bridging sheet region, formed by four anti-parallel β-sheets: β2 and β3, which constitute the stem of the deleted V1/V2 loop; and the β20 and β21 of the C4 region.

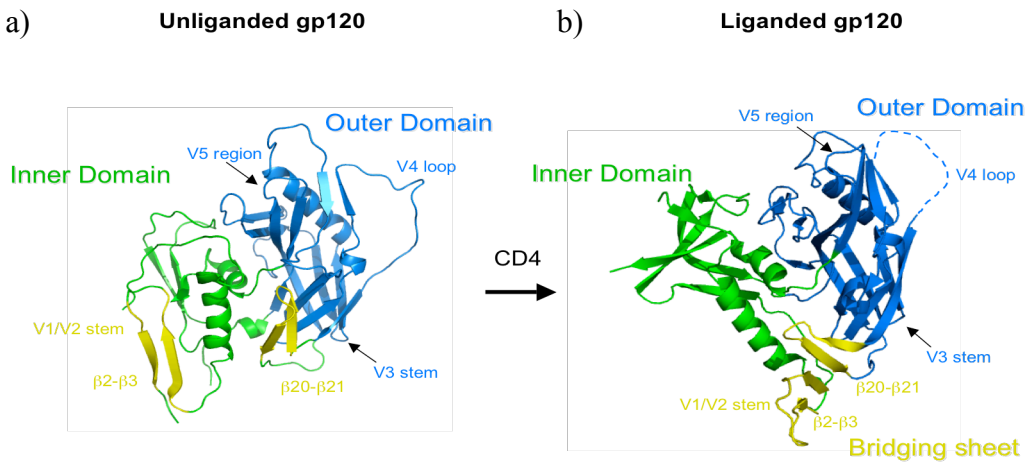
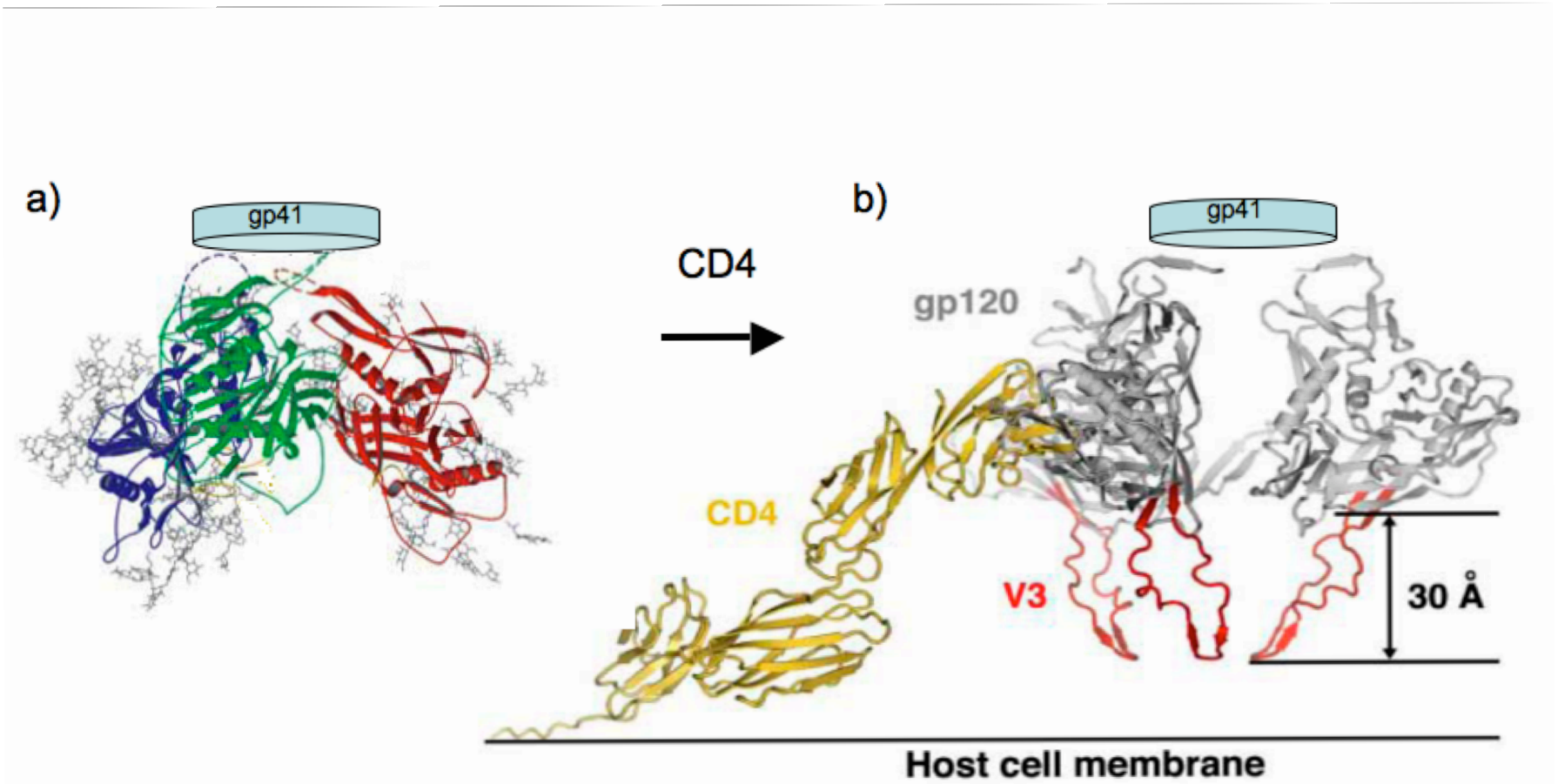


Figure 1.2: Crystal structures of gp120 core in unliganded and CD4-bound conformations. Ribbon diagram of (a) the unliganded SIV gp120 and (b) the HIV-1 liganded gp120 structures are depicted from the CD4 perspective. The structural domains are colored; green for the inner domain, blue for the outer domain and yellow for the bridging sheet. Variable loops stems and the β-sheets that form the bridging sheet are also indicated. Figures were created in PyMol from the PDB data files 2BC4 (Chen et a., 2005) and 1GC1 (Kwong et al., 1998).

More recently the gp120 of an SIV virus was resolved in the absence of CD4. This unliganded gp120 structure is presumed to represent the native state conformation (Chen *et al.*, 2005). In contrast to the outer domain, which has a similar conformation as the liganded form, the inner domain and the bridging sheet revealed a very altered structure, suggesting that binding of CD4 induces radical conformational changes in these regions. The bridging sheet observed in the CD4 bound conformation appears segregated in the native structure (Figure 1.2a), with the β2 and β3 sheets, laying proximal to the inner domain, displaced 20-25 Å from the β20 and β21 loop.

A third gp120 structure was resolved using an HIV-1 gp120 core that included the V3 loop, complexed to CD4 and the Fab X5 (Huang *et al.*, 2005). In this structure the core resembles the liganded conformation. The V3 loop protrudes as an elongated structure from two anti-parallel β-sheets in the outer domain. A disulphide bond between β12 and β13 stabilizes its base, while a long flexible stem extends away from the core ending in a conserved β-turn tip. The trimeric model of this structure predicts the projection of the tip 30 Å towards the cell membrane, consistent with its positioning towards the coreceptor molecule (Huang *et al.*, 2005) (Figure 1.3). However, it is not clear if V3 displays an extended structure in the context of the native oligomer. It has been suggested that the V3 is partially occluded by the V1/V2 loop of the adjacent protomer in the trimeric envelope glycoprotein, and it is only extended after CD4 interaction (Wyatt *et al.*, 1998).

The widely assumed trimeric structure of the envelope complex has recently been visualized using cryo-electron tomography microscopy by two separate groups (Zanetti *et al.*, 2006, Zhu *et al.*, 2006). Both studies have demonstrated similar dimensions and threefold symmetry for the envelope spike, but the actual map images displayed some distinct features (Figure 1.4). The Zhu *et al.* structure presents multiple lobes emanating from the core and tripod-like configuration of gp41, while the Zanetti *et al*. reconstruction



1.3: Models of the envelope trimer in the unliganded and liganded states. The structures are depicted with the viral membrane positioned at the top of the figure above the schematic representation of gp41. (a) The unliganded trimer was modeled from the SIV gp120 core atomic structure by Chen, et al., 2005. Each of the gp120 monomers is illustrated in a different color, with N-linked glycans represented by stick models. (b) The trimeric model of the CD4-bound envelope glycoprotein was obtained by Huang et al., 2005 using the V3-contaning HIV-1 gp120 core structure. The CD4 receptor is depicted in yellow. The V3 loop is shown in red, extending towards the co-receptor molecule on the host membrane.

constitutes a smooth structure with only three clear lobes and a single stem linking with the membrane (Roux and Taylor, 2007). These differences have been attributed to the techniques used for data collection and analysis (Subramaniam, 2006). Despite these limitations, this rapid evolving methodology promises to assist in solving the native structure of the envelope spike in more physiologically relevant environments, *i.e.* in the context of a viral or cell membrane.

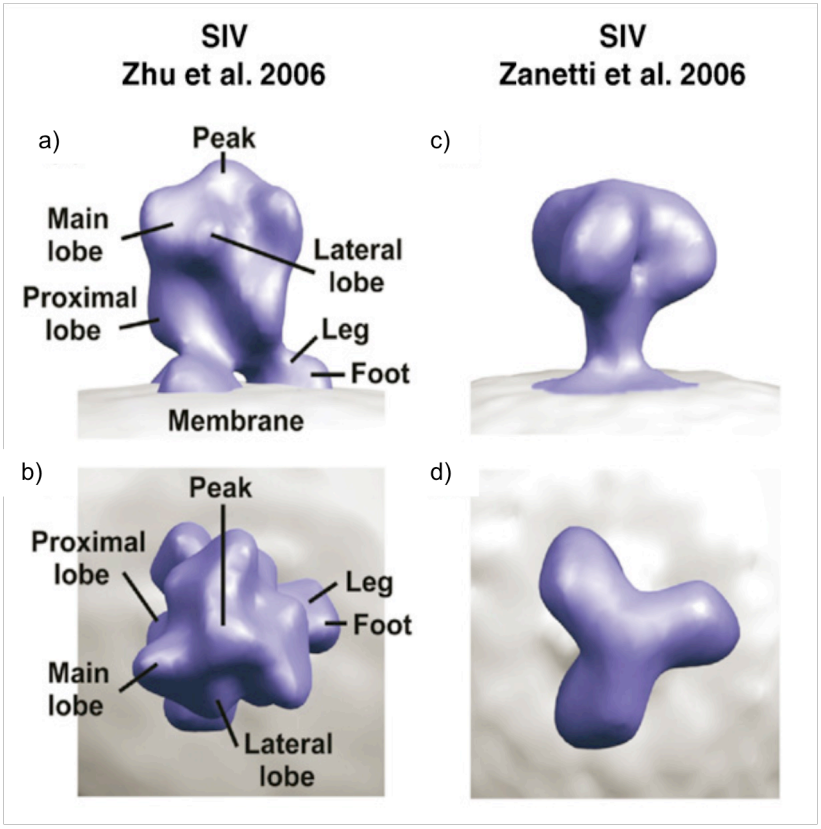


Figure 1.4: Electron tomography density maps of the SIV envelope spike. Side (a, c) and top (b, d) views of the 3D structure of SIV envelope glycoprotein as reported by Zhu et al., 2006 (a, b) and Zanetti et al., 2006 (c, d). Figure adapted from Roux and Taylor, 2007.

1.2.1.2 Functional sites of gp120

1.2.1.2.1 CD4 binding site (CD4bs)

The CD4bs of gp120 constitutes a conformational region suggested to be only apparent in the context of the liganded structure of gp120. It is characterized by a hydrophobic pocket at the interface of the inner domain, the bridging sheet and outer domain, where the side

chain of the Phe43 of CD4 is buried, otherwise known as the "Phe43 cavity" (Figure 1.5). In the unliganded structure many of the residues involved in the interaction with CD4 are distributed around the interface of the inner and outer domain which is lined with hydrophobic residues (Chen *et al.*, 2005). It has been proposed that the CD4 molecule first interacts with the internal face of the conformationally stable outer domains. However, this interaction is not energetically favorable, incurring a substantial drop in entropy (Kwong *et al.*, 2002), and is only stable at the cell surface where the presence of multiple CD4 molecules, binding the trimer simultaneously, increases the avidity of this interaction (Zhou *et al.*, 2007). The binding of CD4 induces large conformational changes in the inner domain, which leads to the formation of the bridging sheet and coreceptor binding site.

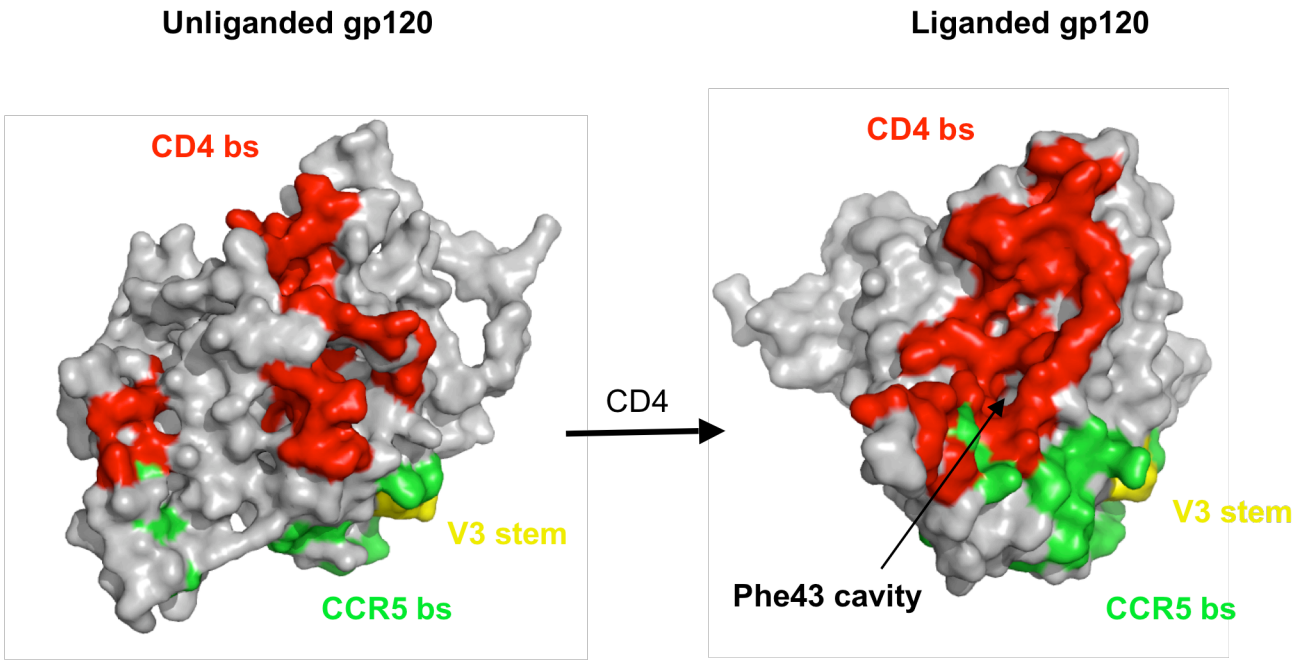


Figure 1.5: CD4 and CCR5 binding surface on unliganded and liganded gp120. Molecular surface diagrams of the unliganded SIV and liganded HIV-1 gp120 core structures are shown from similar orientations. The residues interacting with the CD4 receptor, as documented by Zhou et al., 2007, are shown in red surrounding the Phe43 cavity. The amino acids involved in CCR5 binding, as determined by Rizzuto et al., 1998, are depicted in green in the vicinity of the V3 loop stem, shown in yellow.

1.2.1.2.2 Coreceptor binding site

In addition to CD4 receptors, HIV requires the presence of coreceptor molecules on the surface of the target cells. Most primary isolates use the β-chemokine receptor CCR5 as an entry coreceptor, although some viruses undergo a coreceptor switch mainly to CXCR4 usage. Other minor coreceptors such as CCR1, CCR2b, CCR3, CCR8, CXCR6 (Bonzo/STRL33), Bob/GPR15 and GPR1, have also been shown to mediate virus entry *in vitro*, although their use *in vivo* is less certain (Moore *et al.*, 2004). The V3 loop has been mapped as the major determinant of coreceptor switching, which demonstrates its involvement in the coreceptor-binding site. Other conserved structures of gp120 also form part of this functional region, such as the bridging sheet and the stem of the V3 loop (Rizzuto and Sodroski, 2000, Rizzuto *et al.*, 1998). These residues are segregated in the unliganded gp120 structure and only converge after CD4 binding, to form a conserved pocket that harbors the sulfotyrosines on the N terminus of CCR5 (Figure 1.5). This interaction zips the flexible V3 stem into a rigid β-hairpin (Huang *et al.*, 2007). However, it is not clear if these changes occur before or after the tip of the V3 interacts with the second extracellular loop of CCR5 (Huang *et al.*, 2007).

1.2.2 The gp41 molecule

The gp41 molecule is a transmembrane glycoprotein, that is less variable and less glycosylated than gp120. It interacts non-covalently with gp120 and is responsible for maintaining the trimeric structure of the envelope glycoprotein, although its structure in the native conformation is unknown.

Gp41 constitutes a class I fusion glycoprotein structurally homologous to the fusion proteins of other enveloped viruses such as orthomyxo-, paramyxo-, retro-, filo- and coronaviruses (Dimitrov, 2004). The linear amino acid sequence of gp41 can be divided

into an ectodomain, a very conserved membrane spanning domain and a particularly long cytoplasmic tail (Figure 1.6).

1.2.2.1 gp41 ectodomain and the fusion process

Several important features are present in the ectodomain of gp41 (Figure 1.6). The N-terminus hydrophobic glycine-rich peptide is essential for membrane fusion activity. This fusion peptide is linked, through a flexible polar segment, to a coiled-coil forming amphipathic α-helix (Heptad repeat-1, HR1 or N-helix). A centrally located disulphide-bonded loop connects the HR1 to a second amphipathic α-helix (HR2 or C-helix), which is followed by the membrane proximal external region (MPER).

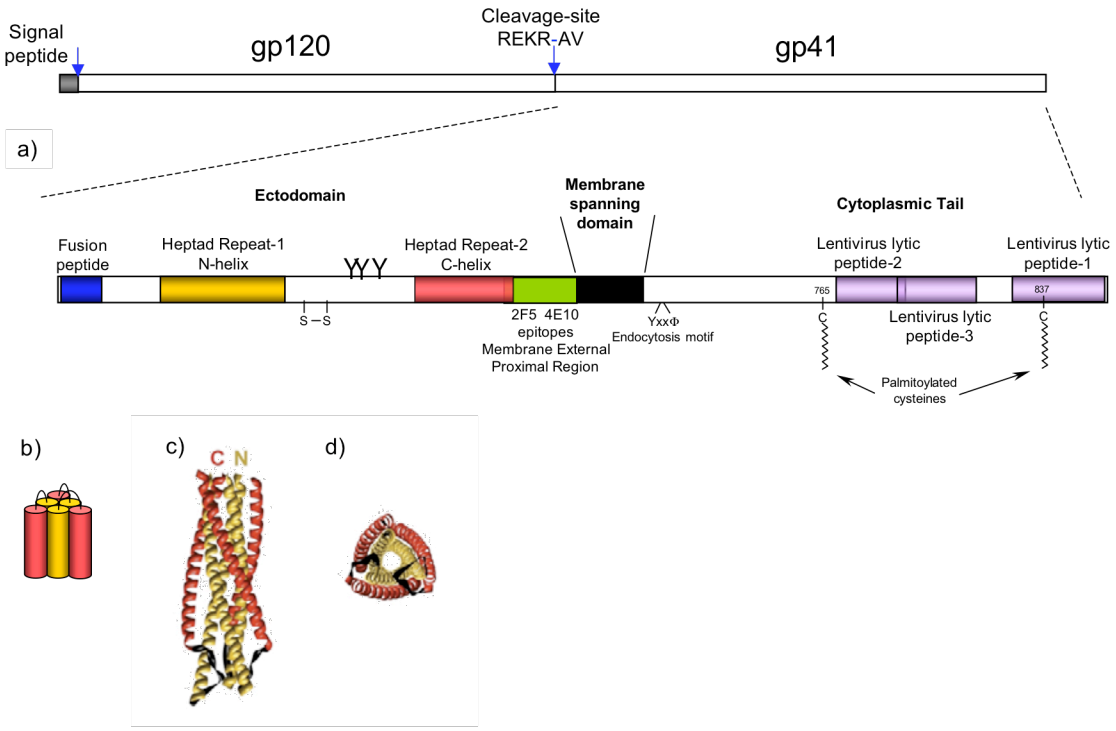


Figure 1.6: Schematic representation of the functional domains of gp41. (a) Functional motifs are indicated in a linear diagram of gp41. The membrane-spanning domain separates the ectodomain from the cytoplasmic tail. The disulphide loop and the glycans in the ectodomain are represented as S-S and Y, respectively. (b) Schematic diagram and (c) side and (d) top view of the atomic model of the six-helix bundle formed by the heptad repeats of gp41 shown in red and yellow (PDB code 1QBZ, Yang, et al.,1999).

In free virions most of the gp41 ectodomain is buried by gp120, protecting this conserved fusion machinery from recognition by the immune system (Figure 1.7a). Only the MPER is

thought to be exposed, as neutralizing antibodies against this region (2F5 and 4E10) are able to bind the native structure before receptor engagement (de Rosny *et al.*, 2004, Zwick *et al.*, 2001). The interface of gp41 with gp120 is not clearly discerned. However, cysteine scanning of the disulphide-bonded region of gp41, and the C1 and C5 regions in the inner domain of gp120 has identified residues that are able to covalently link these subunits (Binley *et al.*, 2000a). Although the gp41 native structure in the prefusogenic state is unknown, experimental evidence suggests that this conformation may be stabilized through interactions with the inner domain β-sandwich (Yang *et al.*, 2003), which prevents the large interactive surfaces of HR1 and HR2 from collapsing into the highly stable six-helix bundle conformation (Weissenhorn *et al.*, 1997). It has been suggested that in this prefusogenic state, the fusion peptide is in close proximity to the MPER (Lorizate *et al.*, 2006) and the HR1 region does not form a coiled-coil structure (Mische *et al.*, 2005).

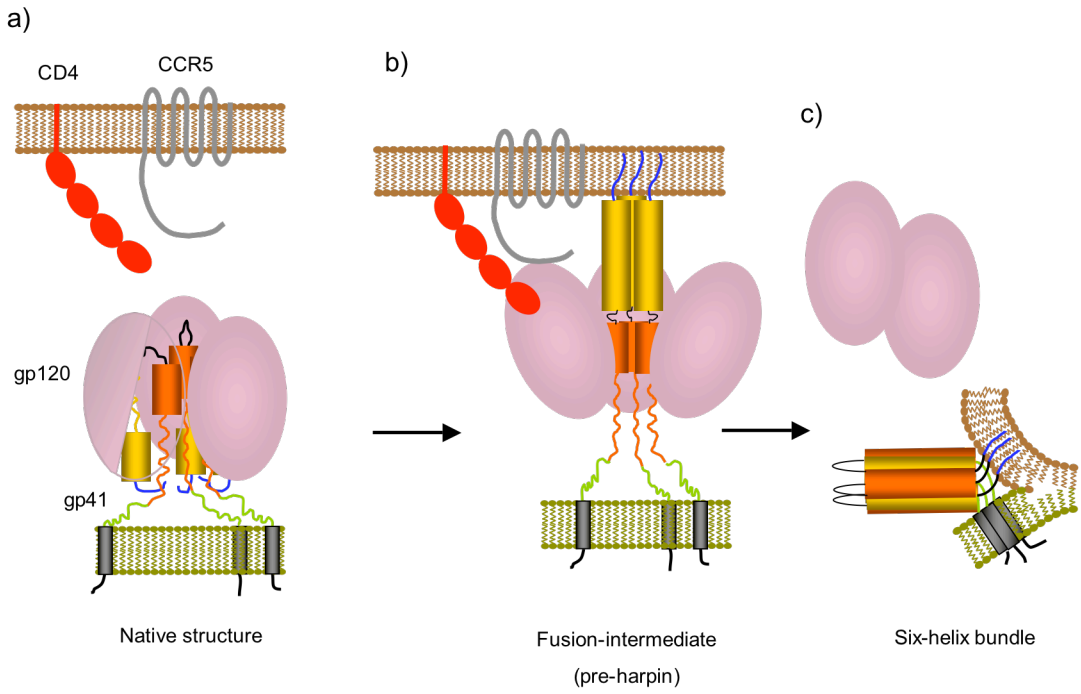


Figure 1.7: Model of the envelope-mediated fusion process. (a) Cartoon showing the envelope trimer before CD4 and co-receptor binding. The gp41 regions are depicted in the same color scheme as in Figure 1.6. The MPER region (green) may be exposed in the native conformation of the envelope. (b) After CD4 and coreceptor engagement, gp41 assumes an extended three-helix rod conformation inserting the fusion peptide (blue) into the host cell membrane. (c) The formation of the metastable six-helix bundle mediates the fusion of the virus membrane into the cell membrane.

Upon CD4 and coreceptor binding the inner domain rearranges, destabilizing the structure of gp41 and inducing the pre-hairpin intermediate state. In this structure, the three gp41 molecules are associated in a trimeric coiled-coil in parallel orientation (Furuta *et al.*, 1998, Jiang *et al.*, 1993, Munoz-Barroso *et al.*, 1998, Wild *et al.*, 1994). The fusion peptide is released and inserted into the target cell membrane (Figure 1.7b). Although the existence of this structure is not clear, it has been extrapolated from the mechanism of the influenza virus hemagglutinin (HA) (Carr and Kim, 1993) and the ability of HR1 binding peptides, such as T-20 (Enfurvitide, DP-178), to trap this conformation and inhibit the fusion process (Furuta *et al.*, 1998).

The fusion-intermediate is brief, with the three HR2 helices rapidly folding into the hydrophobic grove on the surface of the HR1 coiled-coil trimer in an anti-parallel manner, creating a six-helical bundle structure that facilitates the fusion process by bringing the viral and cell membranes together (Figure 1.7c). This conformation is extremely stable, and it is believed that the free energy released during this step contributes substantially to overcome the energy barrier of the membrane fusion process (Chan and Kim, 1998). This post-fusion configuration of the ectodomain of gp41 has been structurally characterized (Weissenhorn *et al.*, 1997), however it is not clear how the transition from the three-helix rod takes place (Chan *et al.*, 1997). It has been suggested that the formation of the six-helical bundle is only completed once the fusion pore has formed (Markosyan *et al.*, 2003).

1.2.2.2 Cytoplasmic tail of gp41

The membrane spanning domain (MSD) precedes an approximately 150 amino acids long segment in the intracellular compartment, known as the cytoplasmic tail (CT). The CT of HIV is, as for most lentiviruses, particularly long compared to other members of the *Retroviridae* family (Hunter and Swanstrom, 1990, Kalia *et al.*, 2003).

Multiple studies have demonstrated the critical role of the CT in virus assembly and

envelope incorporation. Mutations or deletions in the matrix protein (p17) affect envelope incorporation, which can be reversed by compensatory mutations in the CT of gp41, demonstrating the interaction between these two proteins (Freed and Martin, 1996). Several lines of evidence suggest that Gag processing and the fusion machinery of the envelope glycoprotein are connected via the CT. The p55 precursor in the immature virion interacts with the CT of gp41 inhibiting the fusion process, as shown in protease defective viruses, while deletion of the CT disassociates these events (Murakami *et al.*, 2004). This mechanism may avoid early fusion of immature particles that are not able to establish infection.

The CT of gp41 contains a number of highly conserved functional motifs (Figure 1.6). The membrane proximal Tyr-based endocytosis (YxxΦ) motif and the dileucine motif at the C-terminus mediate clathrin-dependent endocytosis, alter intracellular localization, and regulate envelope expression and incorporation in the virion (Byland *et al.*, 2007, Day *et al.*, 2004, LaBranche *et al.*, 1995, West *et al.*, 2002, Wyss *et al.*, 2001, Ye *et al.*, 2004). One or more palmitoylated cysteines (C764 and C837) are implicated in targeting envelope glycoprotein to lipid rafts and mutation of these cysteines to alanine decrease envelope incorporation and infectivity (Bhattacharya *et al.*, 2004, Rousso *et al.*, 2000). Three highly conserved amphipathic α-helices, known as "lentivirus lytic peptide" domains (LLP-1, LLP-2, and LLP-3) are implicated in interacting with the plasma membrane, decreasing bilayer stability, affecting envelope cell surface expression and incorporation into virus particles (Kalia *et al.*, 2003, Piller *et al.*, 2000, Wyss *et al.*, 2005). They have also been described as calmodulin-binding domains that promote Fas-mediated apoptosis of infected cells (Micoli *et al.*, 2006, Srinivas *et al.*, 1993).

1.3 NEUTRALIZING ANTIBODIES

1.3.1 Neutralizing antibody responses in HIV-1 infected patients

In HIV-1 infection, antibodies capable of blocking virus infection *in vitro* develop in almost all individuals, although whether they are able to perform this function *in vivo* is less clear. Their absence during the acute phase of infection, when viral levels are brought under control, suggests that cellular immune responses may be more critical during this period (Moog *et al.*, 1997, Koup *et al.*, 1994). The earliest neutralizing antibodies can be detected after 3-12 months of infection, however, there is considerable variation in the kinetic, magnitude and breadth of this response (Kelly *et al.*, 2005, Moog *et al.*, 1997, Pellegrin *et al.*, 1996, Richman *et al.*, 2003, Wei *et al.*, 2003). In general, the initial neutralization response is narrow, only effective against early autologous viruses (Li *et al.*, 2006a, Moog *et al.*, 1997, Richman *et al.*, 2003) and some T-cell line adapted strains (Pilgrim *et al.*, 1997). Nevertheless, the appearance of neutralization escape variants soon after the autologous response has developed, supports the notion that these antibodies exert immunological pressure on the virus (Wei *et al.*, 2003, Richman *et al.*, 2003). Antibodies capable of neutralizing heterologous viruses develop later in infection, with only a small percentage of chronically infected patients having broadly cross-reactive antibodies against multiple HIV-1 viruses (Braibant *et al.*, 2006, Donners *et al.*, 2002, Pilgrim *et al.*, 1997). The nature of the antibodies in broadly cross-reactive sera, as well as why breadth develops so rarely, is not well understood. It is clear, however, that a threshold of viremia is necessary to induce neutralizing antibodies, demonstrated by their absence in individuals on highly active antiretroviral therapy (HAART) and in "elite controllers" (Bailey *et al.*, 2006, Binley *et al.*, 2000b, Montefiori *et al.*, 2001). In chronic infection, high viremia has been correlated with neutralization breadth (Deeks *et al.*, 2006). On the other hand, rapidly progressing individuals, who lack control over viremia, usually display low neutralizing

antibody titers, but this may be attributed to a general immune suppression (Cecilia *et al.*, 1999, Pilgrim *et al.*, 1997).

A recent study has suggested that neutralizing antibodies might protect from HIV-1 superinfection, as this is more likely to occur during the early phase of infection when these antibodies are absent (Smith *et al.*, 2006). Other studies have shown that maternal neutralizing antibodies can exert powerful protective and selective effects during perinatal HIV-1 transmission with resistant strains establishing infection in infants (Dickover *et al.*, 2006, Wu *et al.*, 2006).

1.3.2 Mechanisms of evasion from neutralizing antibodies

HIV-1 has developed multiple escape mechanisms to avoid neutralization. The shedding of gp120 monomers diverts the immune system towards structures otherwise not found on the native trimer (Wyatt and Sodroski, 1998). The envelope spike is heavily glycosylated, with the poorly immunogenic glycans shielding antibody access to the peptidic structure (Johnson and Desrosiers, 2002). Furthermore, changes in glycan packing yield viruses resistant to the autologous neutralizing antibody response. This neutralization escape mechanism is referred as an “evolving glycan shield” (Wei *et al.*, 2003).

The trimeric nature of the envelope glycoprotein shields conserved regions, while exposing relative amorphous highly glycosylated loop structures. These regions tolerate high levels of variation and therefore can easily escape from neutralizing antibodies (Wyatt *et al.*, 1998). Multiple studies have suggested that the V1/V2 loops cover conserved epitopes involved in the coreceptor binding site of the neighboring protomer (Kwong *et al.*, 2000), as deletion of these variable loops confers sensitivity to antibodies targeting this region (Sullivan *et al.*, 1998, Wyatt *et al.*, 1995). The coreceptor binding site is only transiently exposed after receptor engagement and thus out of antibody reach (Labrijn *et al.*, 2003, Wu *et al.*, 1996). The CD4bs on the other hand is exposed for functional reasons, however, a

distinct type of camouflage, called “entropic masking”, protects it. Binding to this epitope requires the fixation of the otherwise flexible gp120, imposing an entropic barrier for the high affinity antibody binding required for neutralization (Kwong *et al.*, 2002).

1.3.3 Broadly neutralizing antibodies

Despite all these defense mechanisms, a few rare broadly neutralizing monoclonal antibodies (nMAb) have been isolated from HIV-1 subtype B infected individuals. These MAb neutralize many primary isolates from different genetic subtypes, indicating some conserved structures on the envelope glycoproteins. Their epitopes include regions in gp41 (2F5 and 4E10), the CD4bs (b12), and part of the carbohydrate-masked “silent face” of gp120 (2G12). Crystallographic analysis of these antibodies have revealed that they underwent remarkable structural adaptations to attain virus recognition (Burton *et al.*, 2005).

Passive immunization of primates challenged with chimeric simian–human immunodeficiency virus (SHIV) strains has shown that human nMAbs can protect against infection and are effective against intravenous (Baba *et al.*, 2000, Mascola *et al.*, 1999), oral (Hofmann-Lehmann *et al.*, 2001) or intravaginal challenges (Mascola *et al.*, 2000, Parren *et al.*, 2001, Veazey *et al.*, 2003). In cases where transmission occurs they can ameliorate disease by blunting the peak of viremia and lowering the viral set point (Ferrantelli *et al.*, 2007). A recent study in humans showed that in some HIV-infected individuals these nMAbs can reduce the rate of viral rebound following a structured treatment interruption (Trkola *et al.*, 2005). Furthermore, the existence of 2G12 escape variants in some of the treated patients demonstrated that this nMAb was indeed functional *in vivo* (Manrique *et al.*, 2007, Nakowitsch *et al.*, 2005).

1.3.4 Neutralizing antibody epitopes

1.3.4.1 CD4bs: b12 epitope

The neutralizing antibody b12 was obtained as a Fab through a phage display library strategy (Burton *et al.*, 1991). The Fab b12 as well as the IgG1 recombinant MAb derived from it, IgG1b12, occlude the CD4bs on gp120 and prevents CD4 attachment (Burton *et al.*, 1994, Roben *et al.*, 1994). A key element of the CD4bs is a recess that forms a contact site for the Phe43 protruding from a loop of CD4 (Wyatt and Sodroski, 1998). The first crystal structure of IgG1b12 revealed that the protruding heavy chain complementarity determining region 3 (CDRH3) was unusually long, allowing it to access the CD4 binding pocket (Saphire *et al.*, 2001). However, it was only the recent crystallized structure of the Fab b12 in complex with gp120 that clarified the mechanism behind this antibody neutralization. The b12 interactions with gp120 are mainly with residues in the structurally invariant outer domain (Figure 1.8). As a result, the b12 binding site, in contrast to the CD4bs, does not differ considerably from the pre- to post-attachment forms of gp120 (Zhou *et al.*, 2007), explaining the previously shown low entropic cost of this interaction (Kwong *et al.*, 2002).

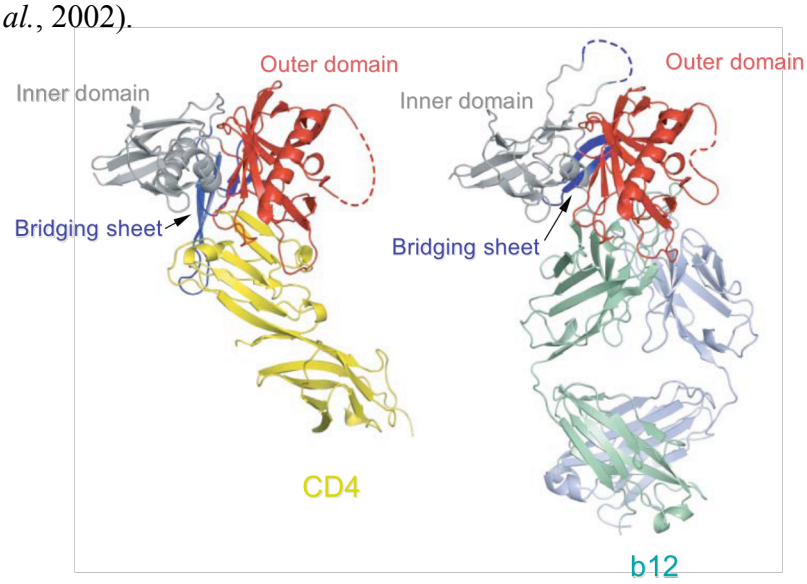


Figure 1.8: CD4 and b12 recognition of gp120. Ribbon diagram of gp120 bound to CD4 (yellow) and b12 (green-light blue) as obtained by Zhou, et al., 2007. The gp120 outer domain (red), inner domain (grey) and bridging sheet (dark blue) are indicated in both structures.

1.3.4.2 Silent face: 2G12 epitope

2G12 recognizes a unique epitope on the surface of gp120 that is not directly associated with the receptor binding sites (Sanders *et al.*, 2002a). Antibody mapping studies using monomeric gp120 showed that 2G12 forms a unique competition group in that no other MAb is able to prevent its binding to gp120 and *vice versa* (Moore and Sodroski, 1996). 2G12 binds to high mannose and/or hybrid glycans, with mannose residues as essential components. Mutagenesis studies have implicated the glycans at positions 295, 332 and 392 in gp120 as being the most critical for 2G12 binding (Sanders *et al.*, 2002a, Scanlan *et al.*, 2002). The X-ray crystallography of this MAb in complex with mannose oligosaccharides, unveiled a unique antibody structure in which the two heavy chain variable domains (V_H) interlock creating a swapped dimer arrangement (Figure 1.9). This creates an extended paratope that allows the recognition of a large oligomannose moiety.

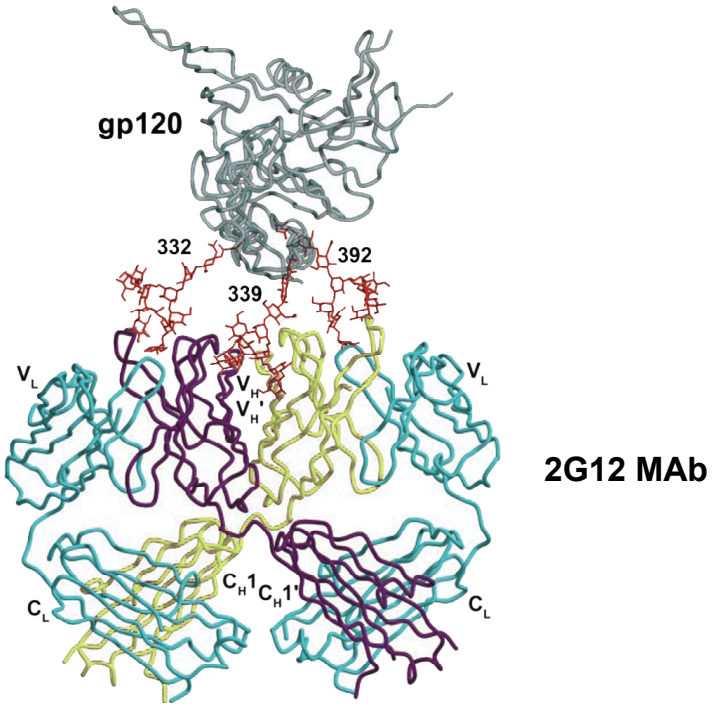


Figure 1.9: Model of the nMAb 2G12 in contact with glycans on gp120. The heavy chains (V_H) of 2G12 (yellow and purple) interlock with the light chains (V_L) (cyan), forming a unique domain-swapped antibody structure. The extended 2G12 idiotope has been modeled to interact with $Man_9GlcNAc_2$ groups (red), attached to the N332 and N392 residues of gp120 (grey) with its primary combining site. The glycan at N339 interacts with a secondary combining site formed by the V_H/V_H' interface. Figure was adapted from Calarese, et al., 2003.

The authors concluded that 2G12 binds to the N-linked glycans at position 332 and 392 in the primary combining sites, with a potential interaction with glycn 339 at the V_H/V_H' interface of the antibody (Calarese *et al.*, 2003). They also proposed that the glycan at position 295 plays an indirect role by preventing further processing of the glycan at 332 and maintaining its oligomannose structure as the one recognized by 2G12.

1.3.4.3 MPER: 2F5 and 4E10 epitopes

2F5 and 4E10 recognize two adjacent highly conserved epitopes in the extreme C-terminal of the gp41 ectodomain (Figure 1.10). This region is particularly attractive for vaccine design because it mediates the viral entry process and is highly conserved between viral strains.

The 2F5 epitope has been mapped to the motif ELDKWA at the end of the HR2 region of gp41 (Muster *et al.*, 1993), where the core residues D664, K665 and W666 are indispensable for antibody recognition (Zwick *et al.*, 2005). Structural data of 2F5 MAb-epitope complexes shows that this region adopts an extended conformation with a type I β-turn at the core of the epitope. Interestingly, the hydrophobic apex of the CDR H3 loop of 2F5 does not interact with the epitope directly (Ofek *et al.*, 2004). It has been suggested that this region mediates interactions with the epitope-proximal viral membrane, explaining early evidence that 2F5 binding was enhanced in the presence of lipids (Grundner *et al.*, 2002).

The nMAb 4E10 recognizes a contiguous epitope at the C-terminus of the 2F5 binding region (Stiegler *et al.*, 2001, Zwick *et al.*, 2001). Mutagenesis experiments have demonstrated that the residues W672, F673 and W680 of the Trp-rich region of gp41 are indispensable for recognition by 4E10 (Zwick *et al.*, 2005). The crystal structure of 4E10 bound to a 13-residue peptide revealed that this epitope assumes an unusual helical conformation. The hydrophobic face of this amphipathic helix is buried in the antibody

combining site, where amino acids W672, F673, I675 and T676 are the key residues in this interaction (Cardoso *et al.*, 2005). Further structural analysis of this epitope has extended it to the motif 672-WFx(I/L)(T/S)xx(L/I)W-680, where x does not play a major role in 4E10 binding (Cardoso *et al.*, 2007).

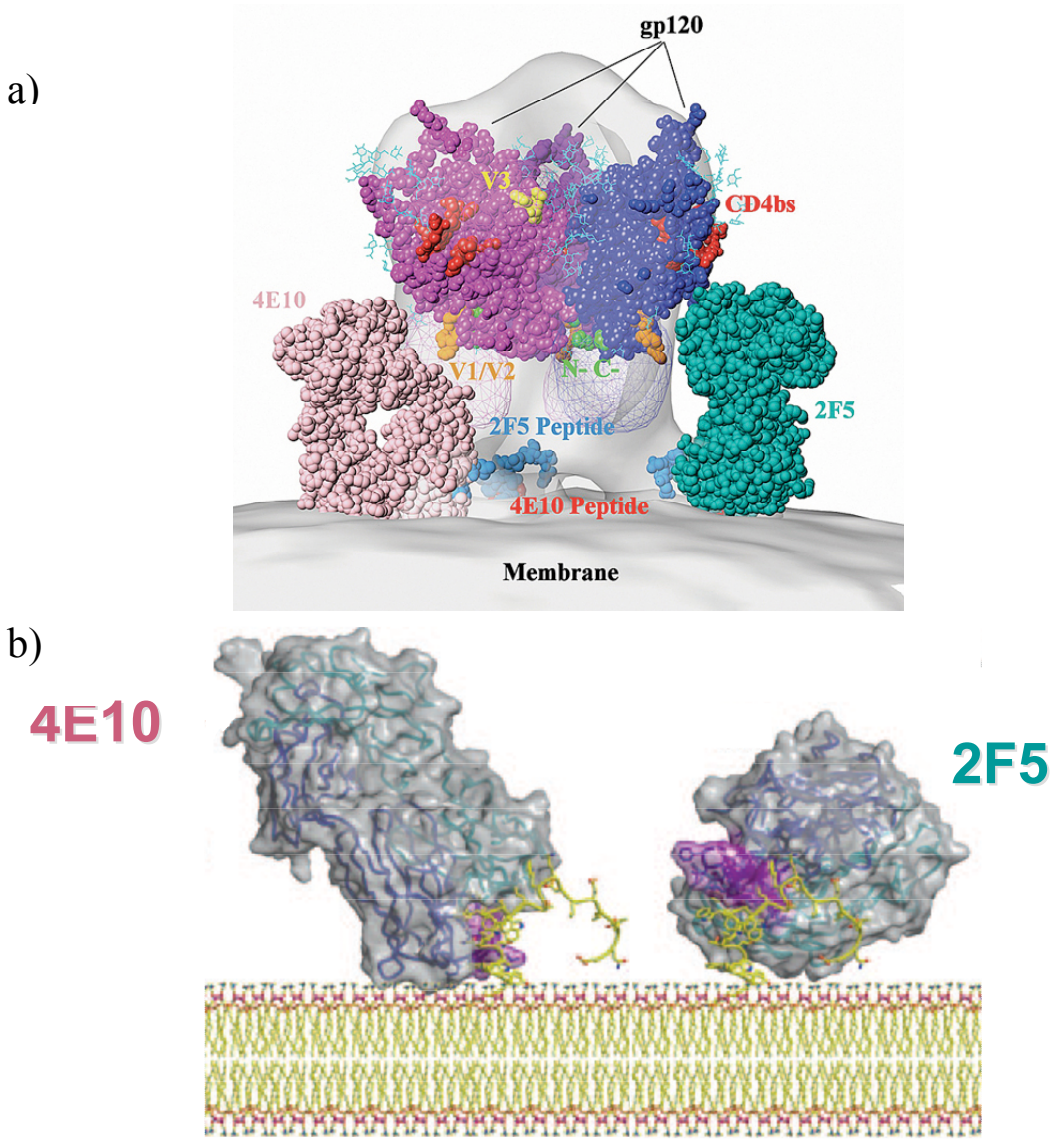


Figure 1.10: Models of the nMAbs 4E10 and 2F5 Fabs bound to their epitopes. The structure of the 4E10 and 2F5 Fabs bound to their peptide epitopes, obtained by Cardoso, et al., 2005 and Ofek, et al., 2004, respectively, are shown in the context of the envelope spike (a) obtained by Zhu, et al., 2006, and (b) in the vicinity of the viral membrane. Figures were obtained from the IAVI report, 2006 (www.iavireport.org) and Burton, et al., 2005

1.3.4.4 V3 loop

In addition to those described above, there are other epitopes able to induce neutralizing antibodies, but in a more limited way. This is the case for the V3 loop of gp120, which was previously considered the principal neutralizing determinant (Palker *et al.*, 1988, Rusche *et al.*, 1988). Later research demonstrated that this was only applicable to TCLA strains, where numerous passages in cell culture rendered these viruses highly sensitive to neutralization by anti-gp120 monoclonal antibodies, patients' sera, and soluble forms of CD4 (Wrin *et al.*, 1995, Verrier *et al.*, 2001, Follis *et al.*, 1998). The mechanism by which sensitivity to neutralizing ligands is acquired is not clear and is manifested only in the context of the functional trimeric envelope spike. Monomeric gp120s derived from either a TCLA strain or a primary isolate exhibit similar affinities for sCD4 (Moore *et al.*, 1991). By contrast the trimeric envelope glycoprotein of TCLA viruses bind the CD4 molecule more efficiently than the primary isolate (Moore *et al.*, 1992, Kabat *et al.*, 1994, Platt *et al.*, 1997). The exposure of epitopes on TCLA viruses may reflect an optimization of the virus-cell interactions, particularly the CD4-gp120, in the absence of selective pressure provided by serum-neutralizing antibodies (Moore *et al.*, 1995).

The V3 loop is less important for primary isolate neutralization, presumably because this region is occluded in the trimeric structure prior to receptor binding. Furthermore, due to the variable nature of this region, most anti-V3 antibodies are isolate-specific. However, a group of these antibodies recognizes conformation-sensitive epitopes on V3 and they are able to neutralize a range of primary isolates. This is the case for the MAb 447-52D, which recognizes the GPGR motif at the tip of the V3 and main-chain atoms along the N-terminal side of the loop (Gorny *et al.*, 1992, Huang *et al.*, 2005, Stanfield *et al.*, 2004). 447-52D neutralizes laboratory strains (Gorny *et al.*, 1992, Gorny *et al.*, 1993) and clinical isolates from various clades (Conley *et al.*, 1994, Nyambi *et al.*, 1998). Although, its activity is

limited to viruses containing the GPGR sequence at the apex of V3 loop, the relatively broad neutralizing activity of 447-52D highlights the existence of conserved structures in the V3 loop and makes this region a potential vaccine target.

1.3.4.5 Coreceptor binding site and/or CD4 induced epitope (CD4i)

The binding of CD4 to gp120 induces conformational changes that lead to the formation of the coreceptor binding site and enhanced binding of a group of antibodies, referred to as CD4i antibodies, such as: 17b, 21c, 23e, 48d, 49e (Xiang *et al.*, 2002), X5 (Moulard *et al.*, 2002), E51 (Xiang *et al.*, 2003) and 412d (Xiang *et al.*, 2005). Crystal structure and mutagenesis data have shown that the epitope recognized by these antibodies overlaps significantly with the highly conserved coreceptor binding site (Huang *et al.*, 2005, Kwong *et al.*, 1998, Xiang *et al.*, 2002, Xiang *et al.*, 2003). In many cases these antibodies mimic the coreceptor molecule by presenting sulfated tyrosine in their CDRH3 (Huang *et al.*, 2007, Huang *et al.*, 2004). CD4i antibodies are commonly found in HIV-infected individuals (Decker *et al.*, 2005) suggesting that this epitope is highly immunogenic. Despite the extremely broad recognition of CD4i antibodies, neutralization is usually impaired. Several studies have shown that virus strains that do not require CD4 for entry are highly sensitive to neutralization by CD4i antibodies (Kolchinsky *et al.*, 2001, Edwards *et al.*, 2001). Moreover, CD4 dependence assures that this immunogenic and conserved region is only exposed after receptor engagement, where the close proximity of the viral and host membranes somewhat restricts the access to this region. This is supported by the fact that small forms of these antibodies, such as Fabs or single chains, display better neutralizing activity (Labrijn *et al.*, 2003). Taken together, these observations preclude this epitope as a good target for vaccine design.

1.4 IMMUNOGEN DESIGN FOR INDUCING NEUTRALIZING ANTIBODIES

The lack of success of monomeric gp120 to induce neutralizing antibodies against primary isolates called for novel vaccine design strategies. Multiple approaches have been tested in the pursuit of an immunogen capable of inducing a broadly cross-reactive neutralization response some of which are described below. While the majority of these candidates have been able to induce neutralizing antibodies, they are usually only successful against viruses that are generally neutralization sensitive or closely related to the antigenic strain.

1.4.1 Native trimeric envelope glycoproteins as immunogens

The observation that neutralization correlates with antibody-binding to the native trimeric envelope glycoprotein (Fouts *et al.*, 1997, Sattentau and Moore, 1995) suggested the use of immunogens that resemble the oligomeric protein on the surface of the virus. Immunization with virus like particles (VLPs), pseudovirions and chemically inactivated viruses, constitute the most direct approaches to present the native trimer to the immune system (Buonaguro *et al.*, 2005, Crooks *et al.*, 2007, Grovit-Ferbas *et al.*, 2000, McBurney *et al.*, 2007, Quan *et al.*, 2007, Race *et al.*, 1995, Wagner *et al.*, 1996). A key issue in these methodologies is the elicitation of antibodies to other cell membrane proteins incorporated in the VLPs, which could confound the interpretation of these results by showing neutralizing activity. Furthermore, the presence of gp120-gp41 monomers and gp41 stumps on these particles could induce non-neutralizing antibodies (Moore *et al.*, 2006). To circumvent these issues it has been suggested that recombinant envelope trimers in proteoliposomes (EnvPL) be used as immunogens (Grundner *et al.*, 2002). This approach was shown to be superior to monomeric gp120 in rabbit immunization experiments (Grundner *et al.*, 2005).

Another strategy to mimic the native envelope structure has been the use of soluble recombinant trimers as immunogens. This approach entails the production and purification

of stable trimeric proteins. A widely followed method has been the use of soluble gp140 with a deleted cleavage site to avoid gp120 shedding (Barnett *et al.*, 2001, Bower *et al.*, 2004b, Srivastava *et al.*, 2002, Yang *et al.*, 2002, Yang *et al.*, 2001, Zhang *et al.*, 2001, Zhang *et al.*, 2007). Further stabilization has been achieved by introducing heterologous trimerization domains such as the yeast transcription factor GCN4 (Yang *et al.*, 2000). The cleavage defective soluble ectodomain construct YU2 gp140 (-/GCN4) has been shown to elicit neutralizing antibodies with some breadth compared to monomeric gp120 in mice, rabbits and guinea pigs (Grundner *et al.*, 2005, Li *et al.*, 2006c, Yang *et al.*, 2001). However, observations that uncleaved forms of the envelope preferentially bind non-neutralizing antibodies suggest that they do not optimally resemble the native glycoprotein (Herrera *et al.*, 2005, Pancera and Wyatt, 2005). As an alternative to preserving the cleavage site, other groups have stabilized the gp120-gp41 interaction by introducing inter-subunit disulphide bonds (SOS) (Binley *et al.*, 2000a, Binley *et al.*, 2002) with further trimerization improvement by introducing a helix-disturbing mutation in the HR1 of gp41 (SOSIP) (Beddows *et al.*, 2007, Beddows *et al.*, 2005, Sanders *et al.*, 2002b). This approach once again rendered antigens superior to monomeric gp120, but they still failed to elicit broadly neutralizing antibodies (Beddows *et al.*, 2007). It is possible that these manipulations do not achieve presentation of the native structure of the envelope glycoprotein. However, immunization experiments with non-stabilized gp140 oligomers were also limited in their ability to induce neutralizing antibodies (Kim *et al.*, 2005).

Strain-specific features may determine the stability of the trimeric structure as well as its capacity to induce broadly cross-reactive neutralizing antibodies (Rademeyer *et al.*, 2007). A recent study showed that the R2 isolate-based uncleaved gp140 oligomer, derived from a patient with broadly cross-reactive neutralizing antibodies, elicited antibodies capable of neutralizing several heterologous viruses from multiple clades and constitutes the most

successful immunogen reported to date (Zhang *et al.*, 2007).

1.4.2 Exposure of cryptic epitopes (CD4i epitope)

Other strategies have involved the exposure of cryptic epitopes, such as the conserved CD4i epitope, by deleting variable loops (Barnett *et al.*, 2001, Gzyl *et al.*, 2004, Kim *et al.*, 2003, Lian *et al.*, 2005, Srivastava *et al.*, 2003) or glycosylation sites (Bolmstedt *et al.*, 2001, Quinones-Kochs *et al.*, 2002) (Nkosi et al. unpublished). Most of these modifications have been done in the context of soluble forms of the stabilized envelope trimer. An alternative to exposing the CD4i epitope has been to co-express gp120 with the CD4 subunit D1D2 (Fouts *et al.*, 2002). This immunogen generated antibodies capable of inhibiting entry of several HIV-1 isolates, but concerns about the induction of anti-CD4 antibodies have been raised. To bypass this difficulty, mimetic forms of CD4 have been used, such as the gp120-M9 construct (Varadarajan *et al.*, 2005); or gp120 liganded to the A32 antibody, which induces CD4i epitope exposure (Liao *et al.*, 2004), but both these methods failed to elicit broadly neutralizing antibodies.

1.4.3 Epitopes of nMAbs as immunogens

Another group of immunogens are based on the conserved epitopes recognized by the broadly nMAbs b12, 2G12, 2F5 and 4E10, aiming to recapitulate their antigenic site (Burton *et al.*, 2004).

The CD4bs is of particular interest as it is exposed on the surface of the virus for functional reasons. However, with the exception of IgG1b12, neutralizing antibodies to this region are not readily induced due to thermodynamic constraints (Kwong *et al.*, 2002, Zhou *et al.*, 2007). The stabilization of the gp120 monomer in a CD4-bound conformation has been proposed as a method to overcome this barrier. Rationalized structure manipulations, such as the introduction of cavity filling mutations and inter-domain disulfide bonds, have

stabilized gp120 conformations that bind CD4 with minimal entropy (Dey *et al.*, 2007, Zhou *et al.*, 2007). Immunization with trimeric gp120-GCN4 with cavity filling mutations induced CD4i antibodies, but showed little improvement in eliciting neutralizing antibodies (Dey *et al.*, 2007). Induction of anti-CD4bs antibodies, such as IgG1b12, has also been pursued through other strategies, such as glycan masking of non-neutralizing epitopes in the gp120 molecule, to focus the antibody response on the b12 epitope (Pantophlet *et al.*, 2003, Pantophlet *et al.*, 2004). While this approach succeeded in masking dominant non-neutralizing epitopes, it did not improve the neutralization response in immunized rabbits (Selvarajah *et al.*, 2005). Assisted by new structural data on the b12 binding site and the biochemical properties of this interaction (Zhou *et al.*, 2007), new strategies to induce b12-like antibodies are under development (Liu *et al.*, 2007, Phogat *et al.*, 2007).

Attempts to reproduce the high mannose clustering recognized by 2G12 using synthetic glycosides and Man9 sugars conjugated to carrier molecules or synthetic scaffolds have been pursued (Dudkin *et al.*, 2004, Geng *et al.*, 2004, Lee *et al.*, 2004, Li and Wang, 2004, Wang *et al.*, 2004). While some of these synthetic oligosacarides inhibit the binding of 2G12 to gp120, their ability to induce neutralizing antibodies has not yet been reported. Another problem with these immunogens is their internal flexibility. Further strategies to enhance the immunogenicity to this epitope were discussed in a recent review by Scanlan *et al.* (Scanlan *et al.*, 2007). They comprise the use of antigenic carriers and adjuvants that can break immunotolerance and the inclusion of antigenic carbohydrates such as rhamnose in these synthetic constructs.

Structural data has shown that the nMAb 4E10 binds to a helical conformation of its epitope. On this basis, stabilization of a peptide containing the 4E10 epitope in a helical conformation has been pursued in the design of a better immunogen (Cardoso *et al.*, 2007).

Further manipulations to hide the non-binding site of this helix, taking membrane interactions into consideration, have also been proposed (Cardoso *et al.*, 2007, Zwick, 2005). Other researchers have designed miniproteins containing the MPER in the context of a proteoliposome or the Hepatitis B surface antigen S1 protein (Ofek *et al.*, 2004). Immunogenicity trials of these variants in a prime-boost regimen with native gp160 trimer have been suggested (Ofek *et al.*, 2004). In a recent study, the MPER was engrafted into the V1/V2 region of gp120, but anti-MPER antibodies were not induced (Law *et al.*, 2007). The knowledge obtained from the 2F5 and 4E10 antibody structures bound to their corresponding epitopes, is currently being exploited in the design of epitope-scaffold immunogens by *in silico* screening of potential carrier proteins (Ofek *et al.*, 2007, Phogat *et al.*, 2007). This inventive methodology promises to produce new candidates soon to be tested in pre-clinical studies.

1.4.4 Multivalent and centralized immunogens

To overcome the genetic diversity of HIV, multivalent or centralized envelope immunogens have been used in preclinical and clinical trials. The first strategy combined the envelopes of multiple HIV-1 clades in one immunization protocol to increase the breadth of the immune responses (Cho *et al.*, 2001, Pal *et al.*, 2005, Rollman *et al.*, 2004, Seaman *et al.*, 2005, Wang *et al.*, 2006). The second approach minimizes epitope diversity by artificially designing envelope genes representing consensus or ancestral sequences of those available in at the Los Alamos HIV Database. This strategy is based on the assumption that these sequences will be more closely related to circulating variants than the circulating variants will be to one another (Nickle *et al.*, 2003). Six centralized immunogens have been generated to date; two consensus M, CON6 (Gao *et al.*, 1996) and CON-S (Liao *et al.*, 2006); subtype-specific consensus ConC (Kothe *et al.*, 2006) and ConB (Kothe *et al.*, 2007), and ancestral Anc1-EnvB (Doria-Rose *et al.*, 2005) and AncC

(Kothe *et al.*, 2006). Of them, the newer consensus, CON-S, was shown to elicit greater neutralization breadth. This suggests that as more sequences are entered into the database, better coverage of envelope variability will be attained.

1.4.5 Other factors that contribute to vaccine design

In addition to antigen design, progress has been made in other areas that contribute to the envelope glycoprotein immunogenicity. DNA prime and recombinant protein boost immunization regimens have been shown to improve the neutralizing antibody responses (Beddows *et al.*, 2005, Law *et al.*, 2007, Lian *et al.*, 2005, Shu *et al.*, 2007, Wang *et al.*, 2005). Other combinations such as DNA prime-VLP boost (Buonaguro *et al.*, 2007a) and DNA prime-viral vector boost (Gomez *et al.*, 2007, Seaman *et al.*, 2007, Shinoda *et al.*, 2006) have also been promising.

Novel adjuvant formulations have been tried in conjunction with newly designed immunogens. The GlaxoSmithKline adjuvants, AS01B, AS02A and AS03 have been shown to enhance the neutralizing titers induced by a soluble trimeric envelope in comparison to the more commonly used Ribi adjuvant (Li *et al.*, 2006c, Zhang *et al.*, 2007). The fusion of the innate activator C3d to an envelope construct proved to enhance immunogenicity (Bower *et al.*, 2004a, Bower *et al.*, 2006). Another formulation involving the co-delivery of GM-CSF DNA was shown to increase avidity of the antibody response (Robinson *et al.*, 2006) and IL-21 and IL-15 gene delivery can increase the level of anti-HIV IgG and the longevity of immune responses (Bolesta *et al.*, 2006). The use of CpG as a mucosal adjuvant was shown to enhance humoral and cellular immune responses in these compartments (Kang and Compans, 2003).

Comparison between the numerous vaccine candidates and immunization strategies has been difficult as various neutralization assays and viral strains have been used in these different studies. This constitutes a major complication in the decision about which

immunogens to advance into clinical trials. Mascola *et al*, suggested the use of cloned envelopes in a pseudovirion neutralization assay in combination with standardized viral panels to evaluate the neutralization responses elicited by candidate vaccines (Mascola *et al.*, 2005). To date, two panels have been assembled, which include viruses from early subtype B and C infections (Li *et al.*, 2005, Li *et al.*, 2006b). Furthermore, a multitier system groups these viruses according to neutralization sensitivity, allowing comparisons of the potencies and breadths of the elicited antibodies (Mascola *et al.*, 2005).

1.5 GENETIC SUBTYPES OF HIV-1 AND NEUTRALIZATION IMMUNOTYPES

An important goal in the development of an effective HIV-1 vaccine is to overcome the extensive genetic heterogeneity of the virus. HIV-1 viruses have been divided in three groups based on their nucleotide sequences: group M (main), group O (outlier), and group N (non-M non-O) (McCutchan *et al.*, 1996). Group M, the largest one, is further divided into genetic subtypes or clades: A, B, C, D, F1, F2, G, H, J and K, plus at least 20 circulating recombinant forms (Buonaguro *et al.*, 2007b). Subtype C is the most prevalent in the world, being common in India and the southern African countries of Botswana, Zimbabwe, Malawi, Mozambique, and South Africa. Subtype B is dominant in North America and Western Europe and has been, until recently, the major focus for vaccine development. Although subtypes are a useful means to categorize HIV-1, the relevance of genetic subtype for vaccine design is uncertain. Genetic subtypes do not readily corresponds to neutralization serotypes and there is no compelling evidence to suggest that HIV-1 positive sera better neutralize viruses from the same clade than viruses from another clade (Kostrikis *et al.*, 1996, Moore *et al.*, 1996, Moore *et al.*, 2001, Nyambi *et al.*, 2000, Weber *et al.*, 1996). However, some have reported stronger intraclade neutralization for subtype A/E (Mascola *et al.*, 1996) or a regional clustering amongst subtype C viruses (Bures *et al.*, 2002). The difficulties associated with finding neutralization immunotypes

have been attributed to the variability of the neutralization titers and specificities in HIV-1 positive sera, as well as the variability of the neutralization sensitivity of the viruses and the high background-to-noise ratio of the methodologies used in these studies. A recent study, which used monoclonal antibodies in a highly reproducible single round neutralization assay, defined five neutralization immunotypes of HIV-1 somewhat associated with genetic clades (Binley *et al.*, 2004). In this case, the absence or presence of some antibody epitopes could be defined in the envelope sequence and correlated with the observed phenotype. The fact that many of the epitopes recognized by these broadly cross-reactive nMAbs have been pursued as targets for vaccine design, underscores the importance of their study in the context of other HIV-1 subtypes.

1.6 OBJECTIVES OF THIS STUDY

The aim of this work was to characterize the neutralizing antibodies epitopes on the HIV-1 subtype C envelope glycoprotein as well as the neutralization response that develops in HIV-1 subtype C infection, in particular the antibody specificities associated with this response.

- Firstly, the sensitivity of HIV-1 subtype C envelope glycoproteins to the broadly neutralizing monoclonal antibodies 2G12, IgG1b12, 4E10 and 2F5 were determined. The presence of the epitope recognized by these antibodies was assessed and compared to the neutralization phenotype.
- Given that subtype C viruses were found to be insensitive to 2G12 neutralization, envelope clones generated in the above study were used in an attempt to reconstitute the 2G12 epitope in subtype C envelopes. This information was further used to assess the antigenic conservation of subtype C envelopes in comparison with their subtype B counterparts.

- The 4E10 epitope is very conserved amongst HIV-1 viruses and an important target for vaccine design. The identification of an HIV-1 subtype C infected individual carrying 4E10 resistant variants allowed the study of the determinants of 4E10 sensitivity in the envelope gene of these viral quasispecies.
- Finally, the kinetics of the neutralization response during natural HIV-1 subtype C infection was analyzed by examining the development of autologous and heterologous neutralizing antibodies in 14 individuals during the first year of infection. In addition, the presence of antibody specificities, such anti-MPER and anti-CD4i neutralizing antibodies, was also assessed.

CHAPTER TWO

INSENSITIVITY OF PAEDIATRIC HIV-1 SUBTYPE C VIRUSES TO BROADLY NEUTRALISING MONOCLONAL ANTIBODIES RAISED AGAINST SUBTYPE B

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Insensitivity of Paediatric HIV-1 Subtype C Viruses to Broadly Neutralising Monoclonal Antibodies Raised against Subtype B

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Abbreviations: IC_{50}, inhibitor concentration; MAb, monoclonal antibody; MTCT, mother-to-child transmission; PBMC, peripheral blood mononuclear cell; PNG, predicted N-linked glycosylation; sCD4, soluble CD4; $TCID_{50}$, tissue culture infectious dose

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ABSTRACT

Background

A Phase I clinical trial has been proposed that uses neutralising monoclonal antibodies (MAbs) as passive immunoprophylaxis to prevent mother-to-child transmission of HIV-1 in South Africa. To assess the suitability of such an approach, we determined the sensitivity of paediatric HIV-1 subtype C viruses to the broadly neutralising MAbs IgG1b12, 2G12, 2F5, and 4E10.

Methods and Findings

The gp160 envelope genes from seven children with HIV-1 subtype C infection were cloned and used to construct Env-pseudotyped viruses that were tested in a single-cycle neutralisation assay. The epitopes defining three of these MAbs were determined from sequence analysis of the envelope genes. None of the seven HIV-1 subtype C pseudovirions was sensitive to 2G12 or 2F5, which correlated with the absence of crucial N-linked glycans that define the 2G12 epitope and substitutions of residues integral to the 2F5 epitope. Four viruses were sensitive to IgG1b12, and all seven viruses were sensitive to 4E10.

Conclusions

Only 4E10 showed significant activity against HIV-1 subtype C isolates, while 2G12 and 2F5 MAbs were ineffective and IgG1b12 was partly effective. It is therefore recommended that 2G12 and 2F5 MAbs not be used for passive immunization experiments in southern Africa and other regions where HIV-1 subtype C viruses predominate.

The Editors' Summary of this article follows the references.

Introduction

Only four broadly neutralising monoclonal antibodies (MAbs) against HIV-1 have been generated to date, all of which were derived from patients with HIV-1 subtype B infection. IgG1b12 recognizes an epitope overlapping the CD4 binding site in the envelope glycoprotein complex [1–5], and 2G12 recognizes a mannose-rich epitope on the silent face of gp120 [6–10]. The 2F5 and 4E10 linear MAbs are located in the membrane-proximal external region of gp41 [11–13]. Passive transfer studies in primates using combinations of these MAbs have provided strong evidence that MAbs are able to control viral replication [14–17] and prevent HIV-1 infection parenterally and through mucosal tissues [18,19]. More recent data have shown that in some individuals with HIV infection, these MAbs can reduce the rate of viral rebound following a structured treatment interruption [20]. Furthermore, oral challenge studies in neonatal macaque monkeys support the use of neutralising MAbs for prevention of virus transmission to human infants [21,22].

Mother-to-child transmission (MTCT) of HIV-1 infection remains a significant problem in developing countries. While the use of single-dose nevirapine, acting to prevent intrapartum transmission, has reduced the number of infections, more potent interventions are needed, particularly to prevent postpartum transmissions. It is estimated that in South Africa alone, approximately 96,000 children with HIV-1 infection were born in 2003 [23]. Passive immunization using neutralising MAbs has been suggested as a strategy to prevent breast milk–borne infections [24,25]. Whether this approach is valid is likely to depend on the efficacy of these MAbs against the targeted viruses.

The most common subtype of HIV-1 infection in southern Africa as well as globally is subtype C (http://www.unaids.org). Results from a previous study indicated that a combination of the MAbs 2F5, 2G12, IgG1b12, and 4E10 successfully neutralised 100% of HIV-1 subtype C isolates tested [26]. However, other studies have shown that 2F5 and 2G12 MAbs are usually ineffective against HIV-1 subtype C viruses, while 4E10 is able to neutralise isolates from all subtypes [27,28]. To further address whether 2G12, 2F5, IgG1b12, and 4E10 are active against HIV-1 subtype C viruses, we tested them in an Env-pseudotyped virus infectivity assay. We chose to use specifically those viruses derived from infants and children who had perinatally acquired HIV-1 infection to determine whether or not these MAbs are effective in vitro as an indication of their potential use for prevention of MTCT.

Methods

HIV-1 Subtype C Viral Isolates

Viruses were isolated from the blood of children with HIV-1 infection by standard co-culture techniques using peripheral blood mononuclear cells (PBMCs) [29,30]. Blood samples were collected from children residing in an orphanage or receiving medical care at the Chris Hani Baragwanath Hospital in Johannesburg between 1999 and 2002 (Table 1) [29]. Informed consent was obtained from either a parent or a guardian of each child at the time of blood collection. This study received ethical approval from the University of the Witwatersrand Committee for Research on Human Subjects (Medical) (Johannesburg, South Africa).

MAbs, sCD4, and Plasma Samples

MAbs were obtained from the National Institutes of Health Reference and Reagent Program (Germantown, Maryland, United States) and the International AIDS Vaccine Initiative Neutralizing Antibody Consortium (New York, New York, United States), and used at a starting concentration of 50 μg/ml. Recombinant soluble CD4 (sCD4) comprising the extracellular domain of human CD4 produced in Chinese hamster ovary cells was obtained from Progenics Pharmaceuticals (Tarrytown, New York, United States), and tested at 50 μg/ml. Two plasma samples (BB12 and IBU21) from blood donors with HIV-1 subtype C infection were tested at a starting dilution of 1:50.

Cell Lines

JC53-bl cells were obtained from the National Institutes of Health Reference and Reagent Program (catalog number 8129). These cells were derived from a HeLa cell clone that expresses CD4, CCR5, and CXCR4 constitutively [31] and contains two reporter genes: firefly luciferase and *Escherichia coli* β-galactosidase under the control of the HIV-1 LTR promoter [32]. The 293T cells used for transfection were obtained from the American Type Culture Collection (Manassas, Virginia, United States). Both cell lines were cultured in D-MEM containing 10% heat-inactivated fetal bovine serum. Cell monolayers were disrupted at confluency by treatment with 0.25% trypsin in 1mM EDTA.

Cloning of Envelope Genes and Production of Pseudovirions

Proviral DNA extracted from in vitro infected PBMCs was used to amplify full-length envelope genes. The 3-kilobase PCR fragments, generated using envA and envM primers [33], were cloned into the pCDNA 3.1-TOPO vector (Invitrogen, Carlsbad, California, United States) and bacterial colonies screened by PCR for insertion and correct orientation using T7 and envM primers. The Env-pseudotyped virus stocks were generated by co-transfecting 2 μg of the *env* encoding plasmid DNA with 3.3 μg of the HIV genomic vector SG3delta *env* (a gift from Beatrice Hahn) into an 80% confluent monolayer of 293T cells in a T-25 culture flask in the presence of 40 μl of PolyFect Transfection Reagent (Qiagen, Heidelberg, Germany). The media was replaced 6–8 h after transfection; 48 h later, culture supernatant containing the pseudoviruses was harvested, filtered (0.45 μm), and stored at −70 °C. The tissue culture infectious doses ($TCID_{50}$) were quantified by infecting JC53-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (30 μg/ml) (Sigma, St. Louis, Missouri, United States). The infection was monitored 48 h later by evaluating the luciferase activity using the Bright Glo Reagent (Promega) following manufacturer instructions. Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin Elmer, Wellesley, California, United States). $TCID_{50}$ was calculated as described [34]. Wells with relative light units greater than 2.5 times the negative control (mock infection) were considered positive for infection.

Single-Cycle Neutralisation Assay

Neutralisation was measured as a reduction in luciferase gene expression after a single-round infection of JC53-bl cells with Env-pseudotyped viruses [35]. Briefly, 200 $TCID_{50}$ of

Table 1. Patient Information and Viral Isolate Characteristics for HIV-1 Subtype C Cloned Envelope Genes

Cloned Env	Date of Sample Collection	Gender	Age	Viral Load (Copies/ml)	Clinical Category[a]	Biotype of Pseudovirus	Env Genetic Subtype	Accession Number of Cloned Env Gene
RP1.12	February 2002	F	1 y	178,830	C	X4	C	DQ447271
RP4.3	March 2002	M	4 mo	>500,000	C	R5	C	DQ447270
RP6.6	March 2002	M	4 mo	>500,000	C	R5	C	DQ447269
COT6.15	May 1999	F	2 y	267,999	C	R5	C	DQ447266
COT9.6	May 1999	M	1 y	>500,000	C	R5	C	DQ447272
TM7.9	September 1999	M	9 y	66,774	B	R5	C	DQ447267
TM3.8	July 1999	F	6 y	11,178	B	R5	C	DQ447268

[a]B, moderately symptomatic; C, symptomatic with an AIDS defining condition.
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pseudoviruses in 50 μl culture media was incubated with 100 μl of serially diluted MAbs, plasma, or sCD4 using D-MEM with 10% fetal bovine serum in a 96-well plate in triplicate for 1 h at 37 °C. MAbs were either tested singly starting at 50 μg/ml (before addition of cells) or in combination also at 50 μg/ml for each MAb. Thus, TriMab contained 2G12, IgG1b12, and 2F5 (50:50:50 μg/ml) and TriMab plus 4E10 contained 2G12, IgG1b12, 2F5, and 4E10 (50:50:50:50 μg/ml). A 100-μl solution of JC53-bl cells (1×10^4 cells/well) containing 75 μg/ml DEAE dextran was added; the cultures were then incubated at 37 °C in 5% CO_2/95% air for 48 h. Infection was monitored by evaluating the luciferase activity. Titres were calculated as inhibitor concentration (IC_{50}) or reciprocal plasma dilution (ID_{50}) values causing 50% reduction of relative light units compared to the virus control (wells with no inhibitor) after subtracting the background (wells without virus infection). IC_{50} values obtained for MAb combinations were compared to MAbs tested singly. The HIV-1 subtype B pseudovirus QH692.42 was included as a positive control, because this virus has been known to be sensitive to all four of the test MAbs [36,37].

gp160 Sequencing

Cloned *env* genes were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, California, United States) and resolved on an ABI 3100 automated genetic analyzer. The full-length gp160 sequences were assembled and edited using Sequencher (version 4.0) software (Gene Codes, Ann Arbor, Michigan, United States).

Results

HIV-1 Subtype C Cloned Envelopes from Paediatric Patients

We cloned complete (gp160) envelope genes from seven HIV-1 subtype C isolates cultured from the blood of children with perinatally acquired HIV-1 infection. Five of these isolates were from rapidly progressing infants (RP and COT) who developed severe clinical symptoms within the first year of life, most of whom died shortly after blood collection (Table 1). Two isolates were from children who had survived for between 6 and 9 y and were moderately symptomatic with illnesses, such as lymphocytic interstitial pneumonitis. All isolates used the CCR5 co-receptor, while viruses from one rapidly progressing infant (RP1) also used the CXCR4 co-receptor and was therefore dualtropic [29]. The Env-pseudotyped virus derived from the latter isolate was able to use only CXCR4 as co-receptor, while the other six pseudoviruses used CCR5 (Table 1). All cloned envelopes were sequenced and compared to the original viral isolate. Phylogenetic analysis indicated that all samples were HIV-1

Table 2. Sensitivity of HIV-1 Subtype C Pseudovirions to Anti-HIV MAbs, sCD4, and Plasma

Env Clone	IC_{50} (μg/ml)[a]							Plasma ID_{50}[b]	
	2G12	IgG1b12	2F5	4E10	TriMAb[c]	TriMAb+ 4E10[d]	sCD4	BB12	IBU21
RP1.12	>45	>45	>45	**13.2**	>50	**8.9**	**16.4**	**28**	<22
RP4.3	>45	**0.9**	>45	**17.1**	**1.6**	**1.0**	**8.4**	<22	**383**
RP6.6	>45	**11.9**	>45	**45.8**	**20.1**	**11.1**	**27.0**	**587**	**1,018**
COT6.15	>45	>45	>45	**3.0**	>50	**0.9**	**8.3**	**153**	**128**
COT9.6	>45	**3.4**	>45	**35.9**	**5.0**	**2.6**	**0.4**	**114**	<22
TM7.9	>45	**0.2**	>45	**34.5**	**0.2**	**0.2**	**7.3**	<22	<22
TM3.8	>45	>45	>45	**21.6**	>50	**13.5**	**26.0**	**218**	**2,399**
QH692.42	**0.8**	<**0.4**	**7.1**	**15.2**	ND	ND	**2.7**	<22	**47**

[a]Concentration of each MAb alone or in combination that achieves 50% neutralisation are in bold.
[b]Reciprocal plasma dilution.
[c]TriMAb: Equimolar combination of 2G12:2F5:IgG1b12.
[d]TriMAb+4E10: Equimolar combination of 2G12:2F5:IgG1b12:4E10.
ND, not determined.
DOI: 10.1371/journal.pmed.0030255.t002

subtype C isolates; sequences from the same individual clustered together with high bootstrap values (unpublished data).

Neutralisation Sensitivity of HIV-1 Subtype C Env-Pseudotyped Viruses to MAbs

The HIV-1 subtype C envelope clones were used to generate Env-pseudotyped viruses by co-transfection with a subgenomic plasmid. These pseudoviruses were tested for their sensitivity to neutralisation by the MAbs IgG1b12, 2G12, 2F5, and 4E10. The MAbs 2G12 and 2F5 failed to neutralise any of the seven HIV-1 subtype C pseudoviruses at 50 μg/ml, whereas the HIV-1 subtype B virus QH692.42 had IC_{50} values of 0.8 and 7.1, respectively (Table 2). The IgG1b12 neutralised four of the seven HIV-1 subtype C viruses as well as the HIV-1 subtype B control. The IC_{50} values of the sensitive pseudoviruses ranged from 0.2 μg/ml to 12 μg/ml, indicating high potency of this MAb. The MAb 4E10 neutralised all the viruses. The IC_{50} values were generally high, supporting the notion that this MAb has broad specificity but lower potency than other MAbs [27].

Neutralisation Using Combinations of MAbs

Synergistic neutralisation among MAbs that recognize different specificities in the envelope glycoprotein has been suggested [38,39], although it has been a controversial topic. We decided, therefore, to test combinations of these MAbs using equimolar concentrations of 2G12, IgG1b12, and 2F5 (TriMAb), and TriMAb plus 4E10. The IC_{50} values in the presence of TriMAb were similar to those for IgG1b12 alone (Table 2), indicating that the activity in TriMAb was probably due to the activity of IgG1b12. When 4E10 was added to TriMAb, it was not surprising that neutralisation of all isolates was achieved, because 4E10 was active against all isolates when used alone at this concentration range.

Analysis of the dose-response curves confirmed the lack of significant synergy among MAbs. Those viruses sensitive to IgG1b12 (RP4.3, RP6.6, TM7.9, and COT9.6) had similar neutralisation curves in the presence of IgG1b12 alone or when tested as part of TriMAb with or without 4E10 (Figure 1A). However, among isolates insensitive to IgG1b12 (COT6.15, TM3.8, and RP1.12), slightly greater potency was observed with TriMAb plus 4E10, compared to 4E10 alone (Figure 1B).

Sensitivity to sCD4 and Polyclonal Anti-HIV Antibodies

Given the relative resistance of the HIV-1 subtype C pseudovirions to neutralisation by MAbs, we chose to test their responses to sCD4 and polyclonal antibodies from individuals with HIV-1 infection. sCD4, which blocks gp120 binding to the CD4 receptor, neutralised all of the pseudovirions (Table 2), indicating that the CD4 binding site is accessible on the pseudotyped envelope glycoproteins. The IgG1b12 binding site overlaps with the CD4 binding site; however, there was no correlation between the ID_{50} values for sCD4 and IgG1b12 in this assay, similar to what others have reported [28,40].

All pseudovirions except TM7.9 were neutralised by one or both of the plasma samples with a wide variation in IC_{50} titres, as is often seen when using polyclonal antibodies, suggesting that these envelopes were not atypical in their ability to be neutralised (Table 2).

Analysis of Amino Acid Sequences Comprising the Neutralisation Epitopes

Sequence analysis of the predicted N-linked glycosylation (PNG) sites at positions 295, 332, and 392, which are critical for the 2G12 epitope, indicated that all HIV-1 subtype C isolates lacked the glycan 295. TM7.9 also lacked the glycan 392 (Table 3). Another site (position 386), reported to play an indirect role in the formation of the 2G12 epitope, was also absent from one of the HIV-1 subtype C envelopes. The HIV-1 subtype B pseudovirus QH692.42 was the only virus possessing all five PNG sites and was the only virus sensitive to 2G12. These data suggest that the lack of the glycan 295 renders isolates resistant to 2G12, as previously suggested [9,10].

The 2F5 epitope is centred on the sequence ELDKWA [11]. Mutagenesis studies have revealed that the amino acid residues DKW are indispensable for the recognition by this MAb [13,41]. In particular, substitutions at residue K665 appear to be the major determinant of resistance [27]. In this study, all HIV-1 subtype C isolates had substitutions at position 665 with the lysine (K) residue replaced by serine (S) or other amino acids (R or N), while the HIV-1 subtype B pseudovirus QH692.42 had no such substitution. These data support the finding that the residue K665 is crucial for neutralisation by 2F5.

4E10 recognizes an epitope containing the sequence NWF(D/N)IT [12,42] at the C-terminal of the 2F5 epitope. Mutagenesis experiments have shown that the residues W672, F673, and W680 are indispensable for recognition by 4E10 [13], while the crystal structure of the Fab 4E10-epitope complex indicates that W672, F673, I675, and T676 are the key residues in this interaction [43]. All the viruses analyzed in this study had a conserved 4E10 epitope (W672, F673, W680), consistent with their phenotypic sensitivity to this MAb.

Discussion

The neutralisation sensitivity of HIV-1 subtype C isolates derived from children appears similar to previously reported sensitivity of isolates from adults with HIV-1 subtype C infection [27,28]. Thus the broadly cross-reactive neutralising MAbs 2G12 and 2F5 are ineffective against both paediatric and adult HIV-1 subtype C viruses, while IgG1b12 potently neutralised approximately 50% of the tested viruses. Only 4E10 showed broad activity against HIV-1 subtype C viruses, although its potency was low. Collectively, these data caution against the use of 2G12 and 2F5 MAbs for passive immunization in areas where HIV-1 subtype C viruses are highly prevalent.

In this study, we have used cloned envelope genes in a single-cycle neutralisation assay, which is a high-throughput assay that, to our knowledge, is rapidly becoming the method of choice for measuring antibody neutralisation [37,44]. Comparative studies have shown a positive correlation between results derived from this assay and the more traditional PBMC-based neutralisation assay (Taylor et al., unpublished data) [37]. However, the 293T-derived pseudovirions were found to be more sensitive to neutralisation by MAbs and serum samples when compared to the uncloned PBMC-derived viruses [27,37]. It has been suggested that this effect is due to the cells used to generate the pseudoviruses [44] and not the nature of the target cells or the clonal nature

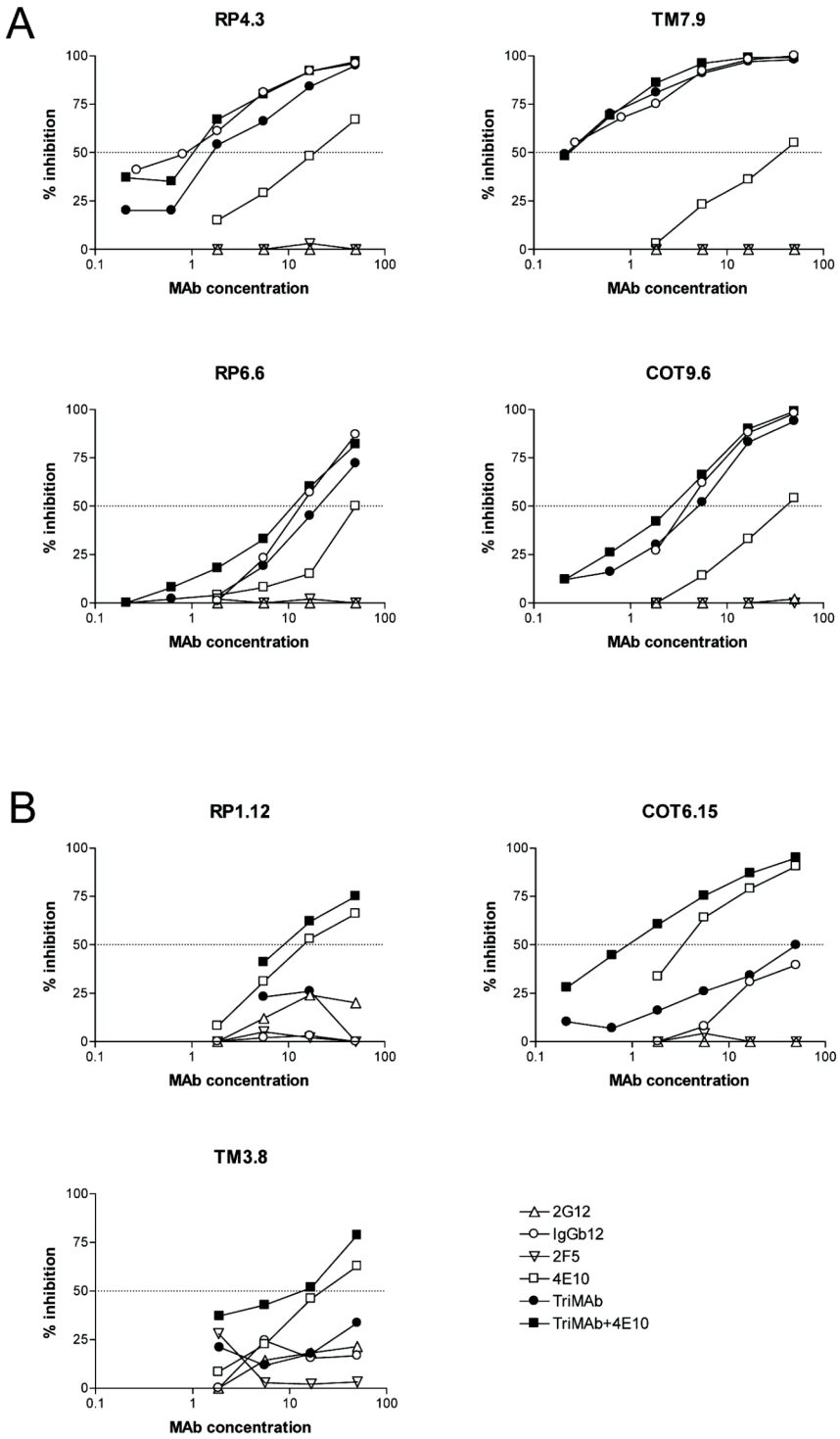

Figure 1. Neutralisation Dose-Response Curves of the MAbs 2G12, 2F5, IgG1b12, and 4E10, Alone and in Combination

The MAb concentrations in the triple and quadruple combination are represented as the concentration of each MAb in the equimolar mix starting at 50 μg/ml. Results are shown as the reduction of virus infectivity relative to the virus control (without MAbs) with 50% inhibition indicated by a dotted line. Note those viruses sensitive to IgG1b12 and 4E10 (A) and those viruses sensitive to 4E10 alone (B).

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Table 3. Amino Acid Sequences of MAb Epitopes in Cloned Subtype C Envelope Genes

Env Clone	2G12 Epitope[a]					2F5 Epitope[b]							4E10 Epitope[b]									
	295	332	392	339	386	662	663	664	665	666	667	668	671	672	673	674	675	676	677	678	679	680
	Nx(S/T)	*Nx(S/T)*	*Nx(S/T)*	*Nx(S/T)*	*Nx(S/T)*	E	L	*D*	*K*	*W*	A	S	N	*W*	*F*	D	I	T	N	W	L	*W*
RP1.12	VCI	***NIS***	***NGT***	***NKT***	***NTS***	A	.	.	***R***	.	N	N	S	.	.	S	.	.	.	.	.	.
RP4.3	ECT	***NIS***[c]	***NNS***	***NDT***	***NTT***	A	.	.	***N***	.	N	S	.	.	.	N	.	.	.	.	.	.
RP6.6	VCT	***NIS***	***NRT***	***NNT***	DTS	A	.	.	***S***	.	N	N	.	.	.	S	.	.	K	.	.	.
COT6.15	VCT	***NIS***	***NTS***	***NRT***	***NTS***	A	.	.	***S***	.	K	N	S	.	.	.	.	.	K	.	.	.
COT9.6	VCT	***NIS***	***NGT***	***NKT***	***NTS***	A	.	***N***	***S***	.	Q	N	S	.	.	S	.	.	.	.	.	.
TM7.9	VCT	***NIS***	NRR	***NKT***	***NTS***	A	.	.	***S***	.	K	N	.	.	.	S	.	S	.	.	.	.
TM3.8	MCT	***NIS***	***NST***	***NKT***	***NTS***	A	.	.	***S***	.	K	N	S	.	.	N	.	S	.	.	.	.
QH692.42	***NCT***	***NLS***	***NST***	***NDT***	***NTT***	.	.	.	.	.	.	.	N	.	.	.	.	.	R	.	.	.

[a]Predicted N-linked glycosylation (PNG) sites are in bold and italic.
[b]Residues crucial for 2F5 and 4E10 MAb activity are in bold and italic.
[c]The PNG is moved two amino acids downstream.
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of the envelope [27,45]. Overall, we can be confident that the observed resistance of HIV-1 subtype C isolates to 2G12 and 2F5 is not due to the use of an Env-pseudotyped virus-based neutralisation assay. Instead, the extra sensitivity of the latter assay might be expected to generate false-positive and not false-negative outcomes.

It has been shown in multiple studies that 2G12 is generally ineffective against HIV-1 subtype C isolates [27,28]. The 2G12 epitope binds a cluster of mannose residues; the absence of an N-linked glycan at position 295 appears to correlate with resistance to this MAb [9,10]. The absence of N295 may prevent the correct processing and presentation of glycans at position 332, affecting antibody binding and therefore neutralisation [7]. A recent study has shown that reintroduction of this PNG site into a subtype C isolate restored binding of 2G12, although sensitivity to neutralisation was not tested [46]. An analysis of 339 HIV-1 subtype C envelope sequences obtained from Los Alamos Database showed that 83% of sequences lacked a glycosylation site at position 295. If the lack of the PNG at position 295 is indeed a cause of resistance to 2G12, then a majority of HIV-1 subtype C viruses would be insensitive to this MAb.

The 2F5 MAb has been shown to have broadly neutralising activity but has minimal efficacy against HIV-1 subtype C viruses [27,28]. An alanine scan over the ELDKWAS epitope defined the motif DKW in positions 664–666 as a determinant for 2F5 recognition [13], although some viruses with this epitope are insensitive to this MAb [27]. However, all viruses with a substitution at residue K665 are resistant to 2F5 [27]. Similarly, in this study, we found that all resistant viruses had a substitution at K665 while the subtype B virus did not. Analysis of 324 sequences in Los Alamos Database showed that the subtype C consensus for the 2F5 epitope is ALDSWA, with only approximately 12% bearing a K at position 665. This suggests that the majority of HIV-1 subtype C viruses will also be resistant to 2F5. However, a geographical clustering of some HIV-1 subtype C variants that may be sensitive to 2F5 due to the presence of the DKW epitope has been suggested [27].

Our data with IgG1b12 agree with other studies in that this MAb is more effective than 2F5 or 2G12 at neutralising HIV-1 subtype C viruses, although IgG1b12 inhibited only approximately 50% of the isolates tested [27,28]. Among sensitive isolates, this MAb is particularly potent and requires very low antibody concentrations for 50% inhibition. Due to the conformational nature of the IgG1b12 epitope, it is difficult to predict resistance to this MAb by simple sequence analysis. Some studies have described neutralisation escape mutations for this MAb, such as a proline-to-alanine substitution in position 369 in the C3 region of gp120 [47,48]. In this study, we found no correlation between the presence of a proline at this position and sensitivity to IgG1b12, which suggests that this escape mutation was specific to the isolate used in the referred study.

The 4E10 epitope appears to be the most broadly cross-reactive MAb described to date, neutralising all viruses so far tested. In previous studies, 4E10 has been shown to neutralise 100% of viruses in a comprehensive panel that included all genetic subtypes of HIV-1 group M and some recombinant forms [27,49]. However, 4E10 is a low-potency antibody generally requiring high concentrations to reduce infectivity by 50%, as seen in this and other studies [20,27]. Whether this is a property of the antibody or inaccessibility of the epitope remains to be determined. The motif WF on the 4E10 epitope was 100% conserved in 324 sequences of this portion of gp41 from HIV-1 subtype C viruses in Los Alamos Database. This suggests that HIV-1 subtype C viruses will be universally sensitive to 4E10.

Some studies have suggested that MAbs can act synergistically to increase neutralisation potency against HIV-1 [26,38,39,50,51]. However, this has been a controversial topic, and isolate dependency has been observed [51,52] with different results obtained with T-cell line-adapted virus and primary isolates [39,50,52]. In this study, we did not observe strong synergy among these MAbs. The combination of the four MAbs neutralised all the tested viruses in agreement with other study results for HIV-1 subtype C isolates [26]. This is likely due to the neutralisation activity of individual MAbs rather than the combined effect of them, because a significant increase in potency was not observed with the mixtures. There may have been a slight synergistic effect for RP1.12, TM3.8, and COT6.15 as demonstrated by increased neutralisation when 4E10 was combined with IgG1b12, 2F5, and 2G12. Such an effect is probably due to IgG1b12, given the

absence of the 2G12 and 2F5 epitopes in these viruses. A more thorough analysis of synergism would require titrating 4E10 against IgG1b12 and evaluating the data based on the Chou-Talalay method [39,53]. It is also possible that the clonal nature of the envelope glycoproteins used in this study precluded the detection of synergism. Some researchers have suggested that the heterogeneity of the virus is the cause of the synergistic effects of some neutralising antibody combinations [54]. However, others have observed no differences between virus isolates passaged in PBMCs and cloned envelope pseudotype viruses [39].

The MTCT of HIV-1 infection is usually associated with transmission of single variants [26]. In this study, four of the cloned envelopes were from children infected for fewer than 12 mo, two of which were infected for 4 mo and therefore represent relatively early variants. Although these clones may not have been the earliest transmitted variants, it is unlikely that earlier variants would differ in their neutralisation sensitivity to these MAbs. We base this assumption on the fact that the MAb sensitivities of viruses from adults with HIV-1 subtype C infection, who would be the source of infection in perinatally infected children, are similar. In addition, we did not observe variation in the susceptibility to neutralisation or in the epitope sequences that can be related to the age of the child: infants and children in this study had identical phenotypic and genotypic profiles. Overall, we feel confident that the MAb neutralisation profiles of the viruses analyzed in this study would be representative of the earliest transmitted variants.

Based on our results, we question the use of MAb combinations that include 2F5 and 2G12 as a prophylactic treatment in regions where HIV-1 subtype C viruses predominate, even if such combinations were to include 4E10 and IgG1b12. In passive immunoprophylaxis studies using a single MAb, protection was not observed even when the challenge strain was successfully neutralised in vitro. Only a combination of at least three MAbs with bona fide neutralisation activity against the challenge strain offered complete protection [17]. Such a combination is not likely to be achievable against HIV-1 subtype C isolates. Furthermore, a recent study using a combination of 2G12, 2F5, and 4E10 for the treatment of individuals with HIV-1 infection has denoted that the ability of 2F5 and 4E10 to affect the virus in vivo is unclear and may require very high serum concentrations of these MAbs [20]. This further questions the use of MAb combinations in which only 4E10 has the potential to be 100% effective.

Overall, we believe that the use of these MAbs to prevent MTCT of HIV-1 subtype C infection is unlikely to be efficacious; therefore, a clinical trial should not be conducted. A recent study has confirmed our viewpoint that these MAbs would have limited benefit when used to prevent MTCT in populations with HIV-1 non-B subtype infection [55]. In addition, recent data have suggested that the MAbs 2F5 and 4E10 react against self-antigens, such as cardiolipin, and the MAb IgG1b12 reacts with double-stranded DNA [56]. Although safety concerns exist surrounding the use of these MAbs for treatment [57], no adverse effects have yet been reported in treated adults [20]. It should be noted that this work remains to be corroborated by others. Nevertheless, if these findings on autoreactivity prove to be true, then the utility of these MAbs for in vivo use is in further doubt, particularly if they are to be used in infants.

The study of the epitopes recognized by these broadly neutralising MAbs contributes to the knowledge necessary for the rational design of an immunogen capable of inducing a broad and potent neutralisation response against HIV-1 infection. Considerable efforts have been invested in designing immunogens based on these epitopes [41]. However, given the subtype constraints of some of these epitopes, new, more broadly occurring epitopes need to be found for the design of vaccines that will be able to elicit an efficient neutralising response against a broad spectrum of HIV subtypes.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for nucleotide sequences of the cloned envelope genes discussed in this paper are DQ447266–DQ447272 (Table 1).

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Author contributions. ESG and LM designed the study. ESG and LM analyzed the data. TM and GG enrolled patients. DM provided reagents and technical advice. ESG, TM, GG, DM, and LM contributed to writing the paper. ESG collected data and performed experiments for the study.

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CHAPTER THREE

N-LINKED GLYCAN MODIFICATIONS IN GP120 OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C RENDER PARTIAL SENSITIVITY TO 2G12 ANTIBODY NEUTRALIZATION

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N-Linked Glycan Modifications in gp120 of Human Immunodeficiency Virus Type 1 Subtype C Render Partial Sensitivity to 2G12 Antibody Neutralization▽

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The monoclonal antibody (MAb) 2G12 recognizes a cluster of high-mannose oligosaccharides on the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 and is one of a select group of MAbs with broad neutralizing activity. However, subtype C viruses are generally resistant to 2G12 neutralization. This has been attributed to the absence of a glycosylation site at position 295 in most subtype C gp120s, which instead is typically occupied by a Val residue. Here we show that N-linked glycans in addition to the one at position 295 are important in the formation of the 2G12 epitope in subtype C gp120. Introduction of the glycosylation site at position 295 into three subtype C molecular clones, Du151.2, COT9.6, and COT6.15, did increase 2G12 binding to all three mutagenized gp120s, but at various levels. The COT9-V295N mutant showed the strongest 2G12 binding and was the only mutant to become sensitive to 2G12 neutralization, although very high antibody concentrations were required. Introduction of a glycosylation site at position 448 into mutant COT6-V295N, which occurs naturally in COT9, resulted in a virus that was partially sensitive to 2G12. Interestingly, a glycosylation site at position 442, which is common among subtype C viruses, also contributed to the 2G12 epitope. The addition of this glycan increased virus neutralization sensitivity to 2G12, whereas its deletion conferred resistance. Collectively, our results indicate that the 2G12 binding site cannot readily be reconstituted on the envelopes of subtype C viruses, suggesting structural differences from other HIV subtypes in which the 2G12 epitope is naturally expressed.

The monoclonal antibody (MAb) 2G12 is a broadly neutralizing antibody that recognizes a unique epitope on the surface of human immunodeficiency virus type 1 (HIV-1) gp120 (39), as no other MAb is able to prevent its binding to gp120 and vice versa (31). Recent studies have shown that 2G12 binds to a cluster of high-mannose sugars, with $\alpha 1 \rightarrow 2$ terminal mannose residues as essential components (36, 37). Furthermore, detailed mutagenesis studies on subtype B have implicated the N-linked glycans at positions 295, 332, and 392 in gp120 as being the most critical for 2G12 binding, with glycans at positions 339, 386, and 448 likely playing an indirect role (36, 37, 39). Crystal structures of Fab 2G12 and its complexes with high-mannose glycosides revealed that the two Fabs assemble into an unusual interlocked V_H domain-swapped dimer (5). Computational modeling based on these crystal structures has suggested that 2G12 likely binds to glycans at positions 332 and 392 in the primary combining sites, with a potential interaction with the glycan at position 339 in the V_H-V_H' binding interface (5). Based on this model, the glycan at position 295 is presumed to play an indirect role by preventing processing of the glycan at 332 and thus maintaining its oligomannose structure (5).

HIV-1 subtype C viruses have been shown to be largely insensitive to neutralization by 2G12 (3, 4, 14). A comparative analysis of HIV-1 subtype C and B sequences contained within the Los Alamos HIV database shows significant differences in the frequencies of an Asn residue at position 295 (88% in subtype B versus 12% in subtype C); the consensus for subtype C viruses at position 295 is a Val residue. These findings have led to speculation that the absence of a glycan at position 295 is responsible for the insensitivity of subtype C isolates to 2G12 neutralization (6, 14, 36). This notion was supported by a recent report showing that reintroduction of a glycan attachment site at position 295 into a subtype C gp120 protein expressed in baculovirus resulted in increased binding of 2G12 (6). However, the neutralization sensitivity of this glycan-enriched gp120 to 2G12 was not investigated.

A number of experimental observations suggest possible antigenic differences between subtype B and C envelope glycoproteins. First, the V3 region of subtype C envelopes is less variable than its subtype B counterpart, as reflected in the lower codon-specific nonsynonymous-to-synonymous-substitution ratio and lower covariability (10, 12). Rather, the gp120 segment downstream of V3 that overlaps the C3 region shows higher variability in subtype C viruses (10, 13). Second, studies on HIV-1 subtype C transmission pairs have shown that recipient viruses have fewer N-linked glycosylation sites and shorter V1-to-V4 regions in the envelope glycoproteins than do donor viruses (7, 41), which has not been observed with subtype B transmissions (9). Finally, natural infection with HIV-1 subtype C typically induces higher titers of autologous neutralizing antibody responses that are less cross-reactive than responses in subtype B-infected individuals (15, 22). Structural differences between the envelope glycoproteins of subtype B and C

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viruses may underlie these subtype-specific patterns of antigenic exposure. In this study, we examine some of the glycan requirements that influence the formation of the 2G12 epitope in the context of subtype C envelopes.

MATERIALS AND METHODS

Plasmids, MAbs, and cell lines. Three HIV-1 subtype C functional envelope clones were used. Du151.2 was obtained from David Montefiori (Duke University), and COT9.6 and COT6.15 were generated previously (14). The pSG3Δ*env* plasmid was obtained from Beatrice Hahn. Soluble CD4 and CD4-immunoglobulin G2 (CD4-IgG2) were generously provided by Progenics Pharmaceuticals, Inc. (Tarrytown, NY). MAbs were obtained from the NIH AIDS Reference and Reagent Program and the IAVI Neutralizing Antibody Consortium. Plasma samples from HIV-1 subtype C-infected individuals (BB12, BB107, and IBU21) were purchased from the South African National Blood Service. The cell line JC53bl-13 was obtained from the NIH AIDS Reference and Reagent Program. 293T cells used for transfection were obtained from the American Type Culture Collection. Both cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA.

Site-directed mutagenesis. Specific amino acid changes in the envelope glycoproteins were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The presence of mutations was confirmed by sequence analysis using an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an ABI 3100 automated genetic analyzer.

Generation of Env-pseudotyped virus stocks. Virus stocks were generated by cotransfecting the Env plasmid with pSG3Δ*env* (40), using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). Fifty percent tissue culture infective doses ($TCID_{50}$s) were quantified by infecting JC53bl-13 cells with serial fivefold dilutions of the supernatant in quadruplicate in the presence of DEAE-dextran (30 μg/ml; Sigma-Aldrich, St. Louis, MO). The infection was monitored 48 h later by evaluating the luciferase activity, using the Bright Glo reagent (Promega, Madison, WI) following the manufacturer's instructions. Luminescence was measured in a Wallac 1420 Victor multilabel counter (Perkin-Elmer, Norwalk, CT). The $TCID_{50}$ was calculated as described previously (18). Wells with relative light unit readings of >2.5 times that of the negative control (mock infection) were considered positive.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Pseudoviruses carrying the wild-type and V295N mutant envelopes were pelleted through a 30% sucrose cushion. Viral proteins were resolved in a Criterion 5% Tris-HCl gradient gel (Bio-Rad Laboratories, Hercules, CA) and blotted onto a polyvinylidene difluoride-nitrocellulose membrane (GE Healthcare Life Science, Piscataway, NJ). The membranes were blocked overnight with 5% milk in Tris-buffered saline, probed with the anti-gp120 antibody D7324, and then visualized with a horseradish peroxidase-labeled anti-sheep antibody (Sigma) in conjunction with an enhanced chemiluminescence detection system (GE Healthcare Life Science, Piscataway, NJ).

MAb binding to gp120 (enzyme-linked immunosorbent assay [ELISA]). gp120 molecules present in the supernatant were captured onto a solid phase via adsorbed antibody D7324. MAbs were bound to gp120 in 1% bovine serum albumin–0.05% Tween 20 in phosphate-buffered saline (PBS). Bound antibodies were detected with alkaline phosphatase-conjugate anti-human IgG (Sigma) and followed by the AMPAK amplification system (Dako Diagnostics Ltd., Glostrup, Denmark).

MAb binding to envelope glycoprotein. Flow cytometric analysis was performed to evaluate the binding of MAbs to the envelope glycoprotein on the surfaces of 293T cells transfected with an *env*-carrying plasmid as described elsewhere (16). 293T cells were transfected at 50% confluence with 5 μg of the *env*-carrying plasmid in a T25 flask. After 48 h, the cells were harvested, washed with PBS, and incubated with 20 μg/ml of MAb in a shaker at room temperate. After 1 h, the cells were washed and fixed in 2% paraformaldehyde–60 mM sucrose–PBS, pH 7.4, for 15 min at room temperature (RT). The cells were washed in 20 mM glycine-PBS (solution A) and incubated in 1% bovine serum albumin–0.05% NaN_3 in solution A (solution B) for 15 min at RT, after which 20 μl of anti-human IgG–fluorescein isothiocyanate (Becton Dickinson, San Jose, CA) was added and incubated for 45 min at RT. After two additional washes with solution B, the stained cells were analyzed with a FACSCalibur flow cytometer. Live cells were initially gated by forward and side scatter. A total of 20,000 live cells were acquired for analysis. MAb binding was evaluated by a shift in the mean fluorescence at 575 nm compared to the negative control fluorescence. The data were analyzed using the Cell Quest program.

Single-cycle neutralization assay. Neutralization was measured as a reduction in luciferase expression after a single-round infection of JC53bl-13 cells with Env-pseudotyped viruses (30). Briefly, 200 $TCID_{50}$ of pseudoviruses in 50 μl was incubated with 100 μl of serially diluted MAbs, plasma, or soluble CD4 in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a 96-well plate, in triplicate, for 1 h at 37°C. One hundred microliters of JC53bl-13 cells (1×10^4 cells/well) containing 75 μg/ml DEAE-dextran was added, and the cultures were incubated at 37°C for 48 h. Infection was monitored by evaluating the luciferase activity. Titers were calculated as the inhibitory concentration (IC_{50}) or reciprocal plasma dilution causing a 50% reduction in the relative light units compared to the virus control level (wells with no inhibitor) after subtraction of the background (wells without virus infection). The subtype B pseudovirus QHO692.42, which is sensitive to neutralization by 2G12 and IgG1b12, was included as a positive control (23).

RESULTS

Introduction of an N-linked glycan at position 295 of gp120 results in increased 2G12 binding but not enhanced virus neutralization. We previously characterized a number of subtype C viruses that were refractory to neutralization by MAb 2G12 (14, 24). In an attempt to reconstitute the 2G12 epitope on subtype C gp120, we first introduced a glycosylation site at position 295 by mutating Val to Asn in three previously studied subtype C envelope clones, namely, Du151.2, COT9.6, and COT6.15. These viruses have glycan attachment sites at positions 332 and 392 (393 in Du151.2) which, together with the glycan at position 295, are believed to be required for the formation of the 2G12 epitope (Fig. 1) (5, 36, 37). The incorporation of an extra glycan in all three viruses was confirmed by an increase in the molecular mass of each mutant relative to the parental gp120 (Fig. 2).

Antibody binding to wild-type and V295N mutant viruses was assessed by ELISA. There were no significant differences in binding levels between the wild-type and V295N mutant envelopes for control MAbs IgG1b12 and A32, indicating that the V295N mutation had not caused a substantial conformational change in the gp120s. In contrast to the case for the control MAbs, a distinct increase in 2G12 binding was observed. 2G12 binding to the COT9-V295N gp120 mutant was particularly pronounced, as a fivefold increase in optical density relative to that with wild-type gp120 was observed. For mutants Du151-V295N and COT6-V295N, there was an ~2-fold increase in 2G12 binding (Fig. 3).

To determine if the increased binding of 2G12 to monomeric gp120 would result in enhanced neutralization, wild-type and mutant envelope genes were cotransfected with an *env*-deficient provirus to produce pseudotyped virus particles. Only the COT9-V295N mutant became sensitive to neutralization by 2G12. However, high antibody concentrations were required (IC_{50}, 69 ± 6 μg/ml) (Fig. 4). No measurable increased sensitivity to 2G12 neutralization was observed for mutant viruses Du151-V295N and COT6-V295N, even at antibody concentrations of up to 100 μg/ml. Du151.2 contains N-linked glycosylation sites, at positions 332, 386, and 448, that form part of the 2G12 epitope. However, Du151.2 lacks glycan 339, which is considered to play an important supportive role (36, 37), and the potential N-linked glycosylation site at position 392 is shifted to position 393, which may impact the binding of 2G12 to the oligomannose cluster.

These data suggest that only on COT9.6 was the 2G12

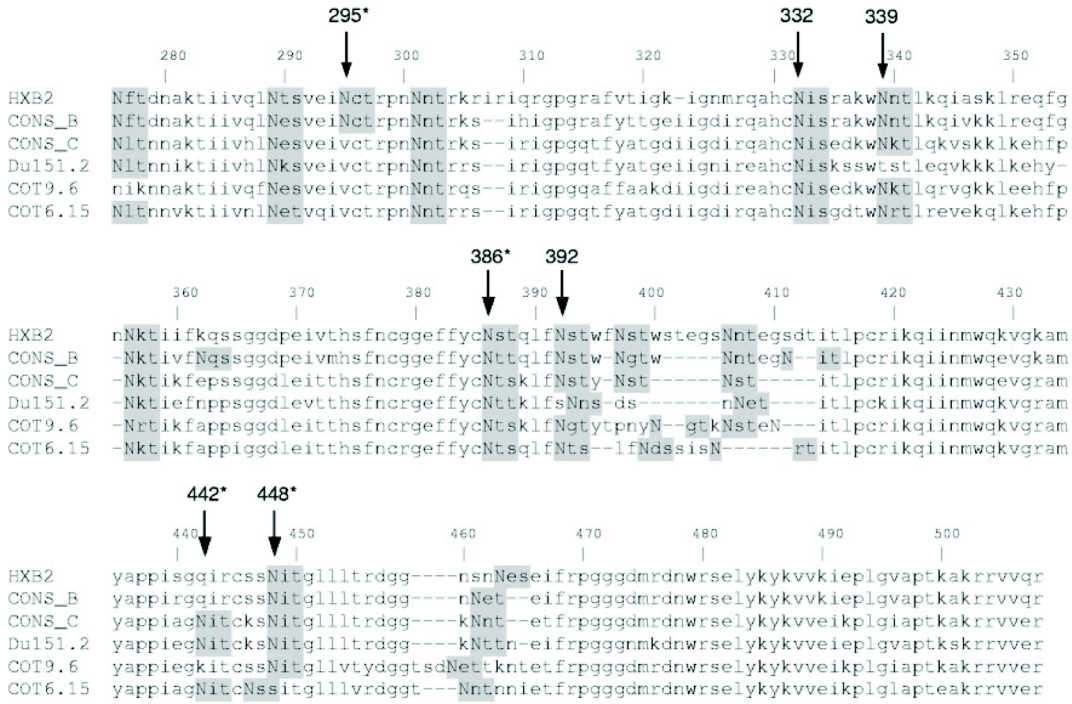

FIG. 1. Amino acid sequence alignment of C2-to-C5 region of gp120s of the three HIV-1 subtype C clones used in this study. Consensus subtype B and C sequences were aligned with the three subtype C envelope sequences and the HXB2 reference sequence. Potential N-glycan attachment sites, determined using N-Glycosite (http://hiv-web.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html), are highlighted in gray. The sites indicated with arrows, except for position 442, have been implicated in the formation of the 2G12 epitope. Those marked with asterisks were analyzed in this study. The N-linked glycosylation site at position 392 in Du151.2 is shifted by one amino acid. The *env* gene nucleotide sequences can be obtained from GenBank under accession numbers DQ447272 (Du151.2), DQ447266 (COT9.6), and DQ411851 (COT6.15).

epitope reconstituted sufficiently well to allow high-affinity binding and neutralization by 2G12. There were no significant differences in neutralization by the IgG1b12 MAb between the mutant and wild-type variants. This demonstrates that the observed variation in 2G12 sensitivity was not the result of a general neutralization-sensitive phenotype of the mutant form and that the introduced mutation had not caused a substantial change in the quaternary conformation of the viral envelope spike (Fig. 4).

Introduction of an N-linked glycosylation site at position 448 further enhances 2G12 neutralization sensitivity. The above results suggested that in addition to the glycan at position 295, further glycans at other positions may be required to form the 2G12 epitope on subtype C viruses. Some studies have suggested that the N-glycan at position 448 may be important for 2G12 recognition (36, 39). Since COT9.6 has an N-linked glycosylation site at position 448, whereas COT6.15 does not (Fig. 1), we hypothesized that the introduction of a glycosylation site at position 448 in COT6.15 and COT6-V295N might allow 2G12 to bind the gp120s of these viruses more efficiently and hence render these viruses sensitive to neutralization by this MAb.

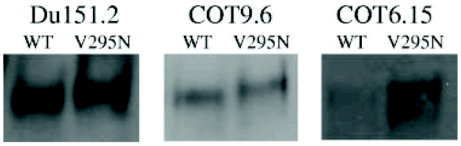

FIG. 2. Introduction of an N-glycan attachment site at position 295 in viruses Du151.2, COT9.6, and COT6.15 leads to an increase in molecular mass. The electrophoretic mobilities of virion-associated gp120s from the wild type (WT) and V295N mutants of Du151.2, COT9.6, and COT6.15 are shown on a 5% sodium dodecyl sulfate-polyacrylamide gel. Western blots were visualized using the antibody D7324, an anti-sheep Ab conjugate, and enhanced chemiluminescence reagents.

Introduction of a glycan attachment site at position 448 in the background of the COT6-V295N mutant (Ser448→Asn) indeed resulted in stronger binding of 2G12 to this gp120, as measured by ELISA and fluorescence-activated cell sorting, than 2G12 binding to wild-type gp120 and the single mutants V295N and S448N (Fig. 5). This increased binding to 2G12 also resulted in viruses that were marginally more sensitive to 2G12 neutralization, although like the case with the COT9-V295N mutant, relatively high antibody concentrations were required. The IC_{50} for mutant virus COT6-V295N/S448N was 65 ± 3 μg/ml. In contrast, wild-type COT6.15 and the COT6-V295N mutant were not neutralized even at antibody concentrations of up to 150 μg/ml (Table 1).

The N-linked glycan at position 442 plays a role in the formation of the 2G12 epitope in subtype C viruses. A further difference between the COT6.15 and COT9.6 sequences is that COT6.15 has a glycosylation site at position 442, whereas in COT9.6 this site is absent due to the occurrence of a Lys

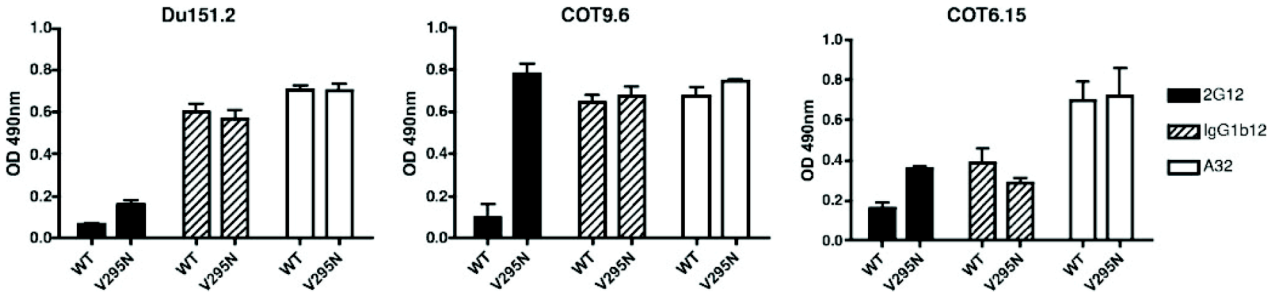

FIG. 3. V295N mutation increases 2G12 antibody binding to subtype C gp120. The binding of MAbs 2G12 (10 μg/ml), IgG1b12 (10 μg/ml), and A32 (10 μg/ml) to the gp120s of Du151.2, COT9.6, and COT6.15, with and without a glycan at position 295, was measured by ELISA. The graphs represent the means for three separate experiments. OD, optical density.

residue (Fig. 1). Sequence analysis of HIV-1 envelope sequences contained within the Los Alamos HIV database (http://www.hiv.lanl.gov/content/index) shows that an N-linked glycan attachment site at position 442 is relatively common among subtype C viruses (54%) but highly unusual among subtype B viruses (3%) (Fig. 1). To examine whether the presence of this glycosylation site influences the formation of the 2G12 epitope, we introduced a glycosylation site at position 442 in the COT9-V295N mutant and eliminated this site in the double mutant COT6-V295N/S448N described above. The COT9-V295N/K422N mutant showed increased binding to 2G12 relative to the wild type and the V295N single mutant (Fig. 6). Accordingly, the corresponding virus was more sensitive to neutralization by 2G12 (IC_{50}, 34 ± 4 μg/ml) (Table 1). Removal of the N-linked glycosylation site at position 442 in virus mutant COT6-V295N/S448N rendered this virus resistant to neutralization by 2G12 (Table 1). Interestingly, only a slight decrease in binding to 2G12 was observed (Fig. 6). These results suggest that the glycan at position 442 indeed contributes to the formation of the 2G12 epitope in the context of subtype C envelope glycoproteins.

Occurrence of the N-linked glycan at position 386 enhances sensitivity to 2G12 in subtype C viruses. We next wished to determine whether the presence of the glycosylation site at position 386 is crucial to the formation of the 2G12 epitope in subtype C viruses, as it is in subtype B (36, 37). Therefore, we eliminated this glycosylation site by mutating Asn386 to Gln in mutant viruses COT9-V295N/K422N and COT6-V295N/S448N, in which the 2G12 epitope had been partially reengineered. The COT9-V295N/K442N/N386Q gp120 mutant showed a threefold reduction in 2G12 binding compared to COT9-V295N/K442N gp120 in ELISA (Fig. 6). For the COT6-

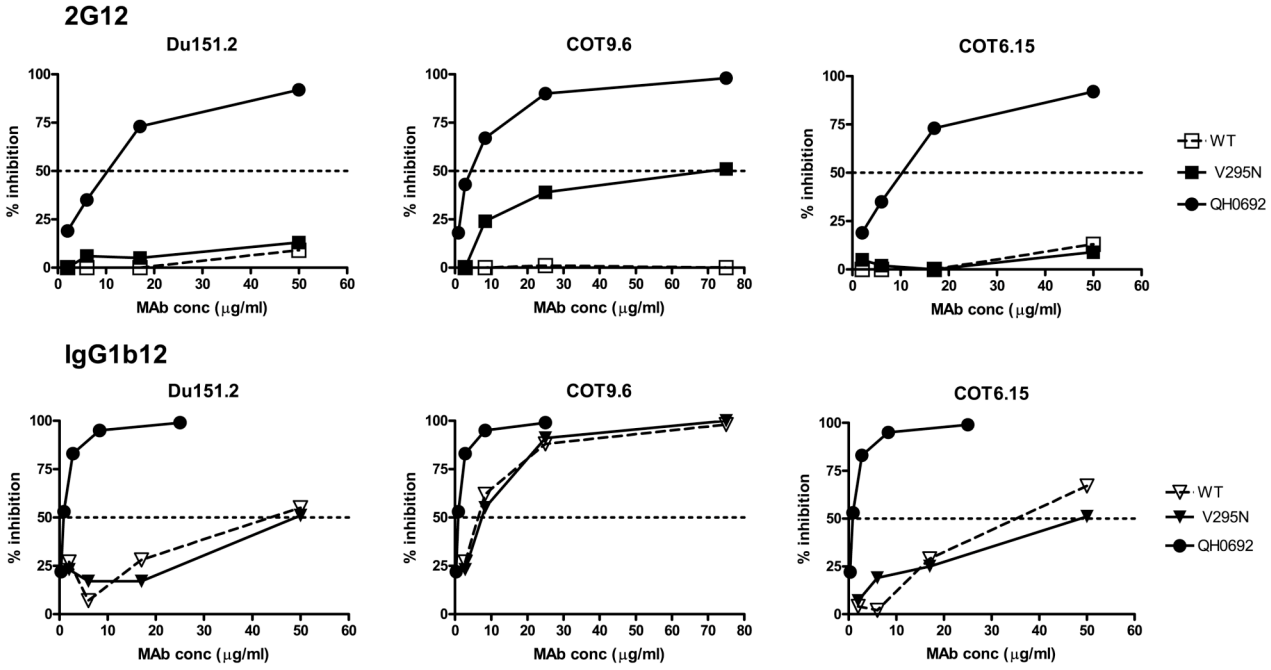

FIG. 4. Neutralization of wild-type and V295N mutant subtype C envelope-pseudotyped viruses by 2G12. Results are shown as reductions of virus infectivity relative to that of the virus control (without MAbs), with 50% inhibition indicated by a horizontal dotted line. MAb IgG1b12 and virus QH0692 (subtype B) were used as positive controls.

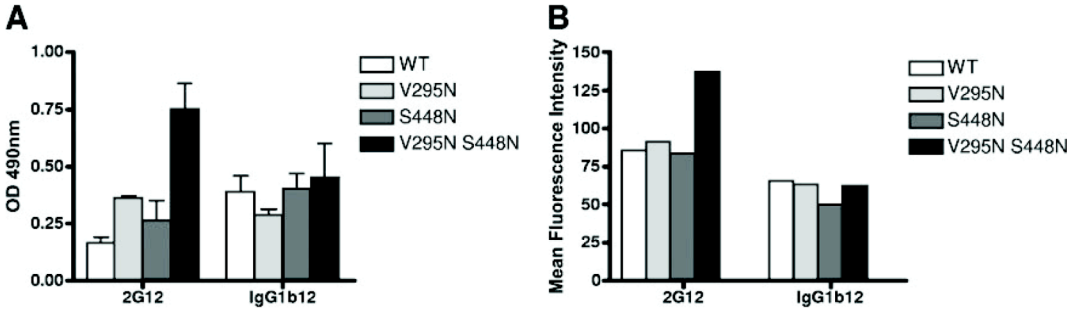

FIG. 5. Impact of V295N and S448N mutations on 2G12 binding to monomeric and oligomeric gp120 from COT6.15. Antibody binding was evaluated by gp120 ELISA (A) and flow cytometric analysis (B) of the envelope protein expressed on the surfaces of transfected 293T cells. ELISA results are the averages for four independent experiments, and fluorescence-activated cell sorting results are from one experiment representative of three. OD, optical density.

V295N/S448N/N386Q mutant, no significant reduction in 2G12 binding was observed. Nevertheless, in both cases, deletion of the glycosylation site at position 386 resulted in resistance to 2G12 neutralization (Table 1). Interestingly, deletion of the glycan at position 386 also resulted in a >10-fold increase in neutralization sensitivity to IgG1b12 for both mutants (Table 1), without a corresponding increase in IgG1b12 binding to monomeric gp120 in ELISA (Fig. 6).

Variation in N-glycosylation also affects neutralization efficiency by MAb 4E10, CD4-IgG2, and HIV-positive plasma. Multiple studies have demonstrated that the introduction or deletion of N-linked glycans in gp120 affects the exposure of certain neutralization epitopes, even when the epitope is not in the immediate vicinity of the site where the mutation is introduced (19, 29). In an effort to determine if other neutralization epitopes may have been affected by these changes in N-linked glycosylation sites, we evaluated the neutralization sensitivities of all the mutant viruses described above to MAb 4E10, CD4-IgG2, and HIV-1-positive plasma samples with broad cross-neutralizing activity from subtype C-infected individuals (BB12, BB107, and IBU21). The anti-gp41 neutralizing MAb 2F5 was not used, as most subtype C viruses, including these three, are insensitive to this MAb (3, 4, 14).

The introduction of a glycan at position 295 in COT6.15 and COT9.6 resulted in a twofold increase in IC_{50} for MAb 4E10 (Table 1), suggesting that this glycan may affect the exposure of the membrane-proximal region, as suggested by others (29). In the case of CD4-IgG2, there was a decrease in sensitivity to neutralization of subtype C viruses in which the glycosylation site at position 386 had been mutated to Gln (Table 1). As mentioned above, N386Q viral mutants showed increased sensitivity to IgG1b12. These contrasting effects on neutralization by CD4 binding site-directed agents suggest that removing the glycan at position 386 may affect the conformation of the CD4 binding site. However, whether this is specific to the Gln substitution was not assessed here and cannot be excluded. The COT9-V295N/K442N/N386Q mutant was also more sensitive to neutralization by two plasma samples, BB12 and BB107, than to the parental virus (Table 1). This difference was not observed for the COT6-V295N/S448N/N386Q mutant and plasma samples BB12 and IBU21. However, these plasmas were better able to neutralize the COT6-V295N/S448N/

TABLE 1. Neutralization of wild-type virus and glycosylation mutants by MAbs, CD4-IgG2, and HIV-positive plasmas from subtype C-infected individuals[a]

Virus and envelope glycoprotein	IC_{50}				ID_{50}		
	2G12	IgG1b12	4E10	CD4-IgG2	BB12	IBU21	BB107
COT9.6							
WT[b]	>100	3.2	3.88	0.08	426	ND	502
295N	**68.7**	3.0	**6.08**	0.08	409	ND	464
V295N K442N	**33.9**	1.7	1.75	0.03	318	ND	460
V295N K442N N386Q	>100	**0.2**	4.59	**0.87**	**759**	ND	**1,000**
COT6.15							
WT	>150	23.3	0.36	0.26	469	391	ND
295N	>150	51.5	**0.75**	0.49	210	303	ND
448N	>150	29.5	0.56	0.67	437	398	ND
V295N S448N	**64.5**	35.2	0.33	0.30	367	393	ND
V295N S448N N442Q	>150	37.8	0.48	0.48	**728**	**557**	ND
V295N S448N N386Q	>150	**1.6**	0.58	**1.32**	325	338	ND

[a] Values in bold indicate significant differences from the parental construct. ND, not determined (COT9.6 was insensitive to IBU21, and COT6.15 was insensitive to BB107, and hence these assays were not performed). IC_{50}, concentration of MAbs or CD4IgG2 that reduces viral infectivity by 50%. ID_{50}, dilution of plasma that reduces viral infectivity by 50%.

[b] COT9.6 naturally harbors N448.

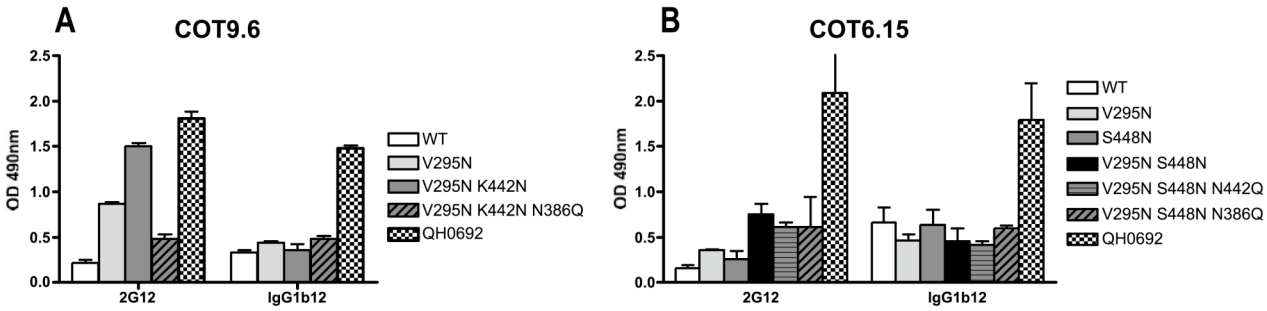

FIG. 6. N-glycosylation at position 442 affects 2G12 and IgG1b12 binding to monomeric gp120. The ability of MAbs 2G12 (10 μg/ml) and IgG1b12 (10 μg/ml) to bind gp120s from the glycosylation mutants of COT9.6 (A) and COT6.15 (B) was assessed by ELISA. The graphs represent the means for three separate experiments. A32 and IBU21 were used to standardize the amount of gp120 bound to the plate (data not shown). The subtype B virus QH0692 was used as a positive control for 2G12 and IgG1b12 binding. OD, optical density.

N442Q mutant, suggesting that the glycan at position 442 may shield certain neutralization epitopes on select subtype C envelopes.

DISCUSSION

This study demonstrates that resistance of subtype C viruses to MAb 2G12 is not due solely to the absence of a glycan at position 295. While this glycan did partially contribute, further reconstitution of the 2G12 epitope required the presence of N-linked glycosylation sites at positions 448 and 442. The glycan at position 448 has previously been implicated in the 2G12 binding site (36, 39), while the glycan at position 442 may be a novel site for the 2G12 epitope in subtype C viruses. However, it should be noted that despite being able to confer 2G12 sensitivity, the antibody concentrations required to achieve neutralization of mutant viruses were at least 10-fold higher than those reported for subtype B viruses (3, 23). Collectively, these results point to possible differences in the structural conformation of subtype C envelope glycoproteins in the region of gp120 that involves the 2G12 epitope.

The N-linked glycan at position 295 is indispensable for the formation of the 2G12 epitope and is the most consistent factor determining the lack of sensitivity to 2G12 neutralization among subtype C viruses. As shown here, the introduction of an N-linked glycan at position 295 resulted in increased binding of MAb 2G12 to gp120s from all three subtype C viruses tested here. A similar finding was reported for a baculovirus-expressed subtype C envelope glycoprotein (6). However, increased binding did not necessarily result in neutralization, as only one of the three mutant viruses examined, COT9-V295N, became sensitive to 2G12. We hypothesize that the conformation of subtype C envelopes precludes the precise formation of the oligomannose cluster necessary for 2G12 binding to the functional envelope spike relative to its occurrence on subtype B viruses. This could be effected by, for example, differences in the processing of glycans between different subtypes. It should be noted that, to date, very little biochemical characterization of N-linked glycans in gp120 has been done (21, 43). More importantly, all analyses have been performed on subtype B gp120s, with no experimental evidence to suggest that subtype C envelopes display the same glycan composition as their subtype B counterparts. Clearly, similar studies using subtype C envelopes will need to be conducted to resolve this issue.

We confirmed that the N-linked glycan at position 448 is a determinant in the formation of the 2G12 epitope, as suggested previously (36, 39). Whether or not this site functions in an isolate-specific manner remains to be elucidated fully. Other studies have not always identified this site as important, including an analysis of 2G12 neutralization escape variants (32, 35).

Our data provide strong evidence that glycosylation sites other than those described for subtype B viruses are required to form the 2G12 epitope on subtype C viruses. Thus, the presence of a glycosylation site at position 442, which is absent in subtype B envelopes, enhanced the binding and neutralization of a subtype C virus by MAb 2G12. This glycan is not in the immediate proximity of the putative 2G12 binding site involving the glycans at positions 332 and 392 (Fig. 7) (5) and therefore may define a new determinant for 2G12 binding in the context of subtype C gp120s. We have not explored if 2G12 binds directly to this glycan or whether it is a complex-type glycan rather than an oligomannose. Nevertheless, it is tempting to speculate that the glycan at position 442 may perform a function similar to that purported for the glycan at position 295 in subtype B viruses, namely, preventing glycosidase trimming of oligomannose clusters (5). Mutants in which this glycan was deleted showed increased neutralization sensitivity to polyclonal HIV-positive sera (Table 1). Given the close proximity of this glycan to the V3 loop (Fig. 7), it may serve to protect the V3 region from neutralizing antibodies. The more frequent occurrence of this glycan among subtype C gp120s than among subtype B gp120s could contribute to the lower levels of variation seen in subtype C V3 sequences (10). However, detailed studies are needed to address this hypothesis directly.

The glycan at position 386 seems to be important for the formation of the 2G12 epitope in subtype C viruses, as it is in subtype B viruses. For both COT9.6 and COT6.15, removal of the glycosylation site at position 386 resulted in resistance to 2G12 neutralization. Unexpectedly, the same mutation showed a marked effect on neutralization by IgG1b12, with a >10-fold increase in sensitivity. The convergence of the 2G12 and b12 epitopes at position 386 was suggested previously based on epitope mapping results (34, 37). This is supported by the recent crystal structure of b12 in complex with a conformation-

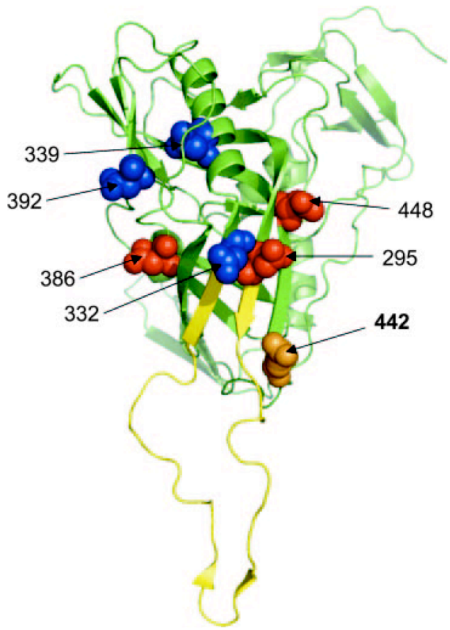

FIG. 7. Location of the Asn at position 442 relative to the location of the V3 loop region and other N-glycans involved in 2G12 binding. The gp120 molecule is viewed with the outer domain facing forward. The structure is rendered as a ribbon diagram, with the V3 loop shown in yellow and the rest of gp120 shown in green. The Asn residues bearing the N-linked glycans that constitute the core of the 2G12 epitope on subtype B gp120 are highlighted in blue as space-filling models, while those supposedly involved in limiting glycosidase trimming are shown in red. The Asn at position 442, identified here as potentially important for the formation of the 2G12 epitope on subtype C gp120, is highlighted in orange. Coordinates were taken from the structure of the $gp120_{JRFL}$ core with V3 ligated with CD4 and X5 (Protein Data Bank accession no. 2B4C). The figure was generated with PyMOL (DeLano Scientific LLC, South San Francisco, CA [http://www.pymol.org]).

ally constrained gp120, in which it is demonstrated that the tip of the CDRH3 domain of b12 interacts with the Asn at position 386 (42). Although Asn386 is not involved in the interaction of CD4 with gp120 (17, 20, 42), we observed a reduction in neutralization sensitivity to CD4-IgG2 when this glycan was deleted, in agreement with previous alanine mutagenesis results (34). Removal of the Asn at position 386 in COT9.6 also resulted in enhanced sensitivity to neutralization by two HIV-positive polyclonal samples. These two plasma samples have broadly neutralizing activity (E. Gray et al., unpublished results), and we are currently exploring whether this is due to the presence of b12-like antibodies, as others have reported (8, 25).

2G12 represents a rare human MAb that shows neutralizing activity against >60% of subtype B HIV-1 isolates in vitro (3, 23). Studies with animals suggest that this antibody is effective at preventing HIV-1 subtype B infection in vivo (1, 27, 28). Recent studies with humans have shown that this MAb can control HIV-1 replication in individuals acutely infected with subtype B viruses (38). However, escape occurs rapidly and is largely confined to glycosylation sites at positions 295, 332, 339, 386, and 392, further supporting the notion that these sites bear glycans that are important for 2G12 binding on subtype B envelopes (26). 2G12-like MAbs are rarely elicited in HIV-infected individuals, probably because the glycans are host derived and thus poorly immunogenic. Nevertheless, the 2G12 epitope is considered a potential vaccine target (33), although its relevance to a subtype C epidemic is doubtful. The absence of the 2G12 epitope in subtype C viruses also hints at possible functional differences between subtype C and B envelope glycoproteins. High-mannose glycans have been implicated in the binding of HIV-1 to DC-SIGN and DC-SIGNR (2, 11), and although these are not restricted to the glycans recognized by 2G12, it would be of interest to determine whether or not the interaction of subtype C viruses with dendritic cells and macrophages is affected by the absence of the 2G12 epitope.

The inability of 2G12 to neutralize HIV-1 subtype C strains, which are now responsible for more than half of global infections, stresses the need to further study and characterize subtype C envelope glycoproteins both antigenically and structurally. Defining the basis for 2G12 resistance might allow for a better understanding of the structure of subtype C envelopes and provide insight into whether a 2G12-like epitope exists on these viruses. More importantly, our results suggest that different neutralization epitopes that might constitute possible targets for vaccine design may be present on subtype C envelopes.

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CHAPTER FOUR

4E10 RESISTANT VARIANTS IN AN HIV-1 SUBTYPE C INFECTED INDIVIDUAL WITH AN ANTI-MPER NEUTRALIZING ANTIBODY RESPONSE

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4E10 Resistant Variants in an HIV-1 Subtype C Infected Individual with an Anti-MPER Neutralizing Antibody Response

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Abstract:

The broadly neutralizing monoclonal antibody (MAb) 4E10 recognizes a linear epitope in the C-terminus of the membrane proximal external region (MPER) of gp41. This epitope is particularly attractive for vaccine design because it is highly conserved amongst HIV-1 strains and neutralization escape *in vivo* has not been observed. Multiple *env* genes were cloned from an HIV-1 subtype C virus isolated from a 7 year old perinatally infected child who had anti-MPER neutralizing antibodies. One clone (TM20.13) was resistant to 4E10 neutralization as a result of an F673L substitution in the MPER. Frequency analysis showed that F673L was present in 33% of the viral variants and in all cases was linked to the presence of an intact 2F5 epitope. Two other envelope clones were sensitive to 4E10 neutralization, but TM20.5 was 10-fold less sensitive than TM20.6. Substitutions at 674 and 677 within the MPER rendered TM20.5 more sensitive to 4E10, but had no effect on TM20.6. Using chimeric and mutant constructs of these two variants, we further demonstrated that the LLP-2 domain in the cytoplasmic tail affected the accessibility of the 4E10 epitope, as well as virus infectivity. Collectively, these genetic changes in the face of a neutralizing antibody response to the MPER, strongly suggested immune escape from antibody responses targeting this region.

Introduction:

The membrane proximal external region (MPER) of the HIV-1 envelope glycoprotein comprises the last 23 amino acids, from residues 660 to 683, of the extracellular domain of gp41 just before the transmembrane domain. This region has attracted a lot of attention in the field of HIV vaccinology due to some particular features: i) it is the target of two of the few broadly neutralizing monoclonal antibodies against HIV-1, namely 4E10 and 2F5, ii) it has been shown to be important in the fusion process and therefore in viral entry (11, 28), and iii) it is a highly conserved linear region among all HIV-1 subtypes.

The MAb 4E10 recognizes an epitope containing the sequence NWF(D/N)IT (30, 38) in the tryptophan-rich region of gp41. Mutagenesis experiments have shown that residues W672, F673 and W680 are indispensable for 4E10 recognition (37). Crystal structures of the Fab 4E10 in complex with a peptide containing the epitope, illustrate that residues W672, F673, I675 and T676 are the key residues in this interaction (7). A more recent study extended the 4E10 epitope to the motif WFx(I/L)(T/S)xx(L/I)W (residues 672-680), where the amino acids marked with an x do not play a major role in 4E10 binding (6). The sequence ELDKWA (residues 663-667) immediately N-terminal to the 4E10 epitope, is the target of the 2F5 MAb (21). Mutagenesis studies had revealed that the amino acid motif DKW is required for recognition by this MAb (37), and structural studies have demonstrated that these three residues are deeply buried in the interface with 2F5 (25). While 4E10 neutralizes viruses from all HIV-1 subtypes, 2F5 fails to neutralize subtype C and some subtype D viruses and this can be directly correlated to changes in the antibody epitope (3, 14).

Despite the high level of conservation of the MPER and its importance in the fusion process, multiple studies have demonstrated that mutations in this region do not necessarily impair viral infectivity (5, 37). It has been proposed that this region is not targeted by the host immune response and therefore is not under diversifying selection pressure (36). Recent studies have addressed the question of whether HIV-1 infection induces the production of neutralizing antibodies that target the MPER. The presence of such antibodies was assessed using a novel strategy where the HIV-1 MPER was engrafted onto an SIV (35) or HIV-2 envelope (F. Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006). These studies

indicated that antibodies with specificities such as 4E10 and 2F5 are rarely produced (J. M. Decker et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006) (35), however, other anti-MPER antibodies were detected in around a third of HIV-1 infected patients (F. Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006). It remains unclear what the effect of such antibodies is on the viral population, as escape variants have not been described.

In this study we characterized HIV-1 subtype C viral quasispecies with different sensitivities to the MAb 4E10. We explored the genetic determinants of these phenotypes as well as the anti-MPER antibody response that developed in the individual from whom this virus was isolated.

Methods:

Cloning of envelope genes and production of pseudovirions: Proviral DNA extracted from *in vitro* infected PBMCs was used to amplify full-length envelope genes using the primers envA and envM (13). The 3Kb PCR fragments were cloned into an expression vector and used to generate Env-pseudotyped viruses as previously described (14).

Envelope sequencing: gp41 was amplified from viral RNA from culture supernatant by nested RT-PCR using published primers (9, 13) and cloned using the TOPO TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA). The gp41 and gp160 clones were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3100 automated genetic analyzer. The sequences were assembled and edited using Sequencher v.4.0 software (Genecodes, Ann Arbor, MI).

Single cycle neutralization assay: Neutralization was measured as a reduction in luciferase gene expression after a single round infection of JC53bl-13 cells with Env-pseudotyped viruses (20). Briefly, 200 $TCID_{50}$ of pseudoviruses in 50 μl were incubated with 100 μl of serially diluted MAbs, CD4-IgG2, plasma or T-20 (Enfurvirtide) in DMEM with 10% FBS in a 96-well plate, in triplicate for 1 h at 37°C. A 100 μl of JC53bl-13 cells ($1x10^4$ cells/well) containing 75 μg/ml DEAE dextran (Sigma-Aldrich, St. Louis, MO) was added and the cultures were incubated at 37°C in 5% CO_2. Infection was determined 48 hours later using the Bright Glo™ Reagent (Promega, Madison, WI). Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin Elmer, Norwalk, CT). Titers were calculated as the inhibitory concentration (IC_{50}) or reciprocal plasma dilution (ID_{50}) causing 50% reduction of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after subtracting the background (wells without virus infection).

Virus infectivity and envelope incorporation: The amount of virus was normalized by the quantity of p24 pelleted through a 20% sucrose cushion for 2 hours at 20,000*g*. JC53bl-13 cells were infected with 10 ng of p24 in the presence of 30 μg/ml DEAE dextran, and cultures were incubated at 37°C in 5% CO_2 for 48 h. Infection was monitored by evaluating the luciferase activity. Envelope incorporation was estimated by Western blot. Viral proteins were resolved in a Criterion 4-15% Tris-HCl Gradient Gel (Bio-Rad Laboratories, Hercules, CA) and blotted onto a PVDF nitrocellulose membrane (GE Healthcare Life Science, Piscataway, NJ). The membranes were blocked overnight with 5% milk in TBS/0.05% Tween 20 and probed with the anti-gp120 (D7324), anti-gp41

(7B2) or the anti-p24 (D7312) antibodies, and developed with an HRP-labeled secondary antibody (Sigma) using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Science, Piscataway, NJ). To determine envelope expression levels, 293T cells were transfected with envelope constructs using the Fugene transfection reagent (Roche, Applied Science, Indianapolis, IN). The cells were harvested after 48 hours and lysated with the non-denaturing lysis buffer, 1% Triton X-100, 300 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4 with Complete protease inhibitor cocktail (Roche). The Western Blot was carried out as described above.

Site directed mutagenesis: Specific amino acid changes in the HIV-1 and HIV-2 envelope glycoproteins were introduced using the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The presence of mutations was confirmed by sequence analysis.

Construction of chimeric envelope glycoproteins: To construct the gp120-gp41 chimeras, the TM20.5 and TM20.6 *env* plasmids were digested with NdeI and the interchanged fragments ligated with T4 DNA Ligase (Invitrogen Corporation). The correct orientation of the cloned fragments was determined by a colony PCR with primers T7 and EnvM. The ectodomain/cytoplasmic tail chimeras were constructing by overlapping PCR spanning the transmembrane domain using the primers: TM20CTfo: CAGATCCGTGAGATTAGTGAGCGGATTCTTAG and EnvM, TM20CTre: CTAAGAATCCGCTCACTAATCTCACGGATCTG and EnvA. The resultant amplicons was cloned into pcDNA3.1D (Invitrogen Corporation, Carlsbad, CA). Chimerism was confirmed by sequencing.

Anti-MPER neutralization assay: This assay was adapted from that previously described by Decker et al. (J. M. Decker et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006) using chimeric HIV-2/HIV-1 MPER constructs (F. Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006) (15). Briefly, 2000 infectious units of virus pre-incubated with plasma/serum at a starting dilution of 1:20 and serially diluted 1:5 in the presence of normal human plasma/serum, was added to 40% confluent JC53bl-13 cells, seeded the day before. Infection was measured 48 hours later by evaluating the luciferase activity as described above.

Nucleotide sequence accession numbers: The GeneBank database accession numbers for the three *env* clones described in this study are EU161643-EU161645.

Results:

Discovery of a naturally-occurring 4E10 resistant virus: While characterizing functional envelope genes from HIV-1 subtype C viruses isolated from children (14) we identified an envelope clone, TM20.13 that was resistant to the MAb 4E10. This was the result of an F to L mutation at position 673, which is known from mutagenesis studies to confer resistance to 4E10 (37). However, this phenotype has not been previously described in patient samples and analysis of sequences in the Los Alamos HIV database suggested that this mutation is very rare in group M of HIV-1. This clone was derived from a virus isolated from a 7 year old female who was infected perinatally and at the time of blood collection had a CD4 count of 334 cells/μl and a viral load of 34,310 copies/ml. Although this patient had lymphocytic interstitial pneumonitis she was considered to be a slow progressor (8). The virus TM20 was isolated on donor PBMC and used the CCR5 coreceptor with a V3 sequence typical of subtype C viruses (8). Only a single sample from this patient was available. Attempts to obtain additional samples either from this individual or from her mother were unsuccessful.

Frequency of the F673L mutation in the TM20 virus isolate: To determine the frequency of the F673L mutation in this cultured virus isolate, we analyzed the gp41 sequences of 43 molecular clones. The molecular clones were generated from 3 independent RT-PCR reactions from the viral RNA to ensure that the mutation was not introduced by PCR error. The mutation F673L was observed in 14 of the 43 (33%) molecular clones sequenced (Figure 1). In total 6 different quasispecies were identified. Interestingly the F673L mutation was always present in conjunction with the mutation S665K in the 2F5 epitope, as well as a group of associated mutations within the HR2 domain and the cytoplasmic tail of gp41 (Figure 1 and data not shown).

Generation and characterization of functional TM20 envelope clones: Full-length envelope genes were amplified from the TM20 isolate and cloned into an expression vector to generate functional clones. Three envelope clones were pseudotyped and characterized for their sensitivity to the broadly neutralizing MAbs 4E10 and 2F5, and the entry inhibitor T-20 (Enfuvirtide, Fuzeon, DP178) (Figure 2A). As predicted from the sequence analysis, the clone TM20.13 was not neutralized by 4E10, but it was sensitive to 2F5. The other two clones were sensitive to 4E10 and resistant to 2F5, but TM20.6 (IC_{50}=1.5 μg/ml) showed at

least 10-fold higher sensitivity to 4E10 than the clone TM20.5 (IC_{50}=17.5 μg/ml). There were no differences in the neutralization sensitivity of the three clones to T-20, which binds to the HR1 region of gp41. In addition, all clones showed similar sensitivity to the HIV-1 positive plasma samples BB12 and BB107 (Figure 2B), suggesting that the increased sensitivity of the clone TM20.6 to 4E10 was not due to a generally neutralization sensitive phenotype. All three clones were resistant to the contemporaneous autologous serum (below 50% neutralization), which is typical in HIV-1 infection.

Sequence analysis of functional clones from TM20: The full-length envelope genes of the three functional clones were sequenced (Figure 3). The genetic variation observed between the clones was consistent with the 7 year duration of infection; however the extent of variability from the C1 to C3 region of the gp120 was surprisingly low. Since these clones were amplified from *in vitro* grown virus, we cannot exclude the possibility that quasispecies bearing different sequences in this region of the envelope existed *in vivo*. The clone TM20.13 had a F673L mutation in the 4E10 epitope as well as the DKW motif intrinsic to the 2F5 epitope, which correlated with the observed phenotypes. TM20.5 and TM20.6 were identical in the gp41 ectodomain, except for two amino acids in the MPER at positions 674 and 677. Multiple other differences between the clones TM20.5 and TM20.6 were observed along the gp120 and the cytoplasmic tail of gp41. All three clones had conserved HR1 and V3 regions which are involved in T-20 sensitivity (10, 27), consistent with the observed equivalent sensitivity to this compound.

Changes in the MPER are partially responsible for the differential sensitivity of TM20.5 and TM20.6 to 4E10: As noted above, TM20.5 was 10-fold less sensitive to 4E10 than TM20.6. These two clones differed in two amino acids in the 4E10 epitope (Figure 3). In order to determine if these residues were associated with 4E10 sensitivity we introduced the mutations D674N and K677N into TM20.6 by site-directed mutagenesis. These changes did not render the TM20.6 mutant more resistant to 4E10 neutralization (Figure 4). Conversely, when the mutations N674D and N677K were introduced in TM20.5 the 4E10 IC_{50} dropped 3-fold suggesting that in some contexts these positions may have an effect on antibody binding affinity.

4E10 neutralization of envelope chimeras: These apparently discordant results suggested

that regions outside the MPER might influence the sensitivity to 4E10. We therefore constructed chimeras in which large regions of the envelope gene were interchanged between the TM20.5 and TM20.6 clones (Figure 5). For both clones, the gp120 subunit had very little to no effect on the neutralization sensitivity indicating that the major determinants of 4E10 sensitivity were in the gp41 region. When the cytoplasmic tail from TM20.6 was combined with the ectodomain of TM20.5, which included gp120 and the external region of gp41, the resulting chimera Ecto5-CT6 was around 4-fold more sensitive than TM20.5. Conversely, Ecto6-CT5 was 3-fold less sensitive than TM20.6. Altogether these results suggest that while the major determinants of 4E10 sensitivity are in the MPER, the cytoplasmic tail can have a tangible impact on the exposure of this epitope.

Mutations in the LLP-2 domain affect sensitivity to neutralization: The cytoplasmic tail sequence of clones TM20.5 and TM20.6 differed by ten amino acid residues (Figure 3), four in the lentivirus lytic peptide-2 domain (LLP-2). Frequency analysis showed that the LLP-2 sequence of TM20.6 was present in 17% of the quasispecies while 36% had the TM20.5 LLP-2 sequence. To determine if variations in LLP-2 were responsible for the changes in 4E10 sensitivity in the cytoplasmic tail chimeras, we exchanged the LLP-2 regions of TM20.5 and TM20.6 by introducing the mutations E783A, T784I, G789V and T792L in TM20.5 (TM20.5 LLP2-6) and the converse changes in TM20.6 (TM20.6 LLP2-5). We tested all constructs for their sensitivity to neutralization by 4E10 and T-20 as well as IgG1b12 and CD4-IgG2 which bind to gp120 and block interactions with CD4.

As observed for the chimera Ecto5-CT6, the mutant TM20.5 LLP2-6 became more sensitive to neutralization by 4E10 when compared to the parental TM20.5 virus (Figure 5). Similarly, these changes in the LLP-2 explained the decrease in sensitivity to 4E10 in the chimera Ecto6-CT5 when compared to the parental TM20.6. Thus, in both cases, the four amino acids in the LLP-2 domain accounted for the altered changes in sensitivity to 4E10 neutralization. These changes also had a minor effect on the neutralization sensitivity for IgG1b12 (Figure 5) and CD4-IgG2 (data not shown) but had no effect on T-20 neutralization.

In order to look at the combined effects of changes in the MPER and LLP-2 we introduced these four mutations into the two MPER mutants to form TM20.5 MPER-6 LLP2-6 and

TM20.6 MPER-5 LLP2-5. For TM20.5 the resultant clone showed a 9-fold increase in neutralization sensitivity to 4E10 and the IC_{50} titer decreased to a similar level to that obtained for the TM20.6 clone (Figure 5). The opposite mutant, TM20.6 containing the MPER and LLP-2 of TM20.5, became 7-fold more resistant to neutralization, but the IC_{50} titer remained slightly lower than for TM20.5 (11.3 versus 17.5 μg/ml), suggesting that perhaps other areas of the envelope may influence this phenotype. These double mutants showed similar sensitivities to IgG1b12 and CD4-IgG2 as the corresponding LLP-2 only mutants corroborating that the MPER plays no role in determining sensitivity to these reagents.

Effect of the gp120 and cytoplasmic tail switching on envelope incorporation and viral infectivity: The LLP-2 domain is a highly conserved cationic amphipathic α-helix with calmodulin-binding properties (29), that is involved in cell-to-cell fusion (17, 33), neutralization sensitivity (16), envelope cell surface expression (4), envelope incorporation into virions and virus infectivity (24). To explore the mechanism behind the effect of the cytoplasmic tail on 4E10 neutralization sensitivity, we compared the viral infectivity and envelope incorporation of the two TM20 clones and their chimeras.

Chimeras bearing the cytoplasmic tail from TM20.6 (ie gp120(5)-gp41(6) and Ecto5-CT6) displayed low infectivity similar to the parental virus (Figure 6A). The TM20.5 cytoplasmic tail conferred an infectivity advantage to the variants carrying it (ie gp120(6)-gp41(5) and Ecto6-CT5). Furthermore, the LLP-2 mutants of TM20.5 displayed the same reduced infectivity as the Ecto5-CT6, while TM20.6 LLP-2 mutants, like Ecto6-CT5, were more infectious (data not shown). This suggested that the increased infectivity conferred by the TM20.5 cytoplasmic tail was largely determined by four amino acids in LLP-2.

The levels of envelope incorporation into virus particles and envelope expression were determined by Western Blot. The virus input was standardized by using equal quantities of p24 antigen, which was confirmed by p24 detection on the blots. The amount of envelope incorporated into virions was assessed by staining with the anti-gp120 antibody D7324 and the anti-gp41 MAb 7B2 to detect gp160 (Figure 6B). In addition, envelope transfected cells were lysed and the levels of envelope expression were determined. Surprisingly, the less infectious TM20.6 clone showed greater envelope expression and incorporation into

virions than the clone TM20.5. This appeared to be determined by the gp120 as all chimeras containing TM20.6 gp120 showed higher levels of envelope irrespective of the gp41 sequence. Thus, the differential neutralization sensitivity between the clones could not be explained by the levels of envelope on virus particles. These results agree with other studies showing that the LLP-2 region can affect viral infectivity without changes in envelope incorporation (17, 24).

Evaluation of anti-MPER neutralizing antibodies in the sera from TM20: The variability within the MPER among the quasispecies and the concomitant variations in 4E10 sensitivity, suggested the presence of immunological pressure targeting this region. To determine if this was the case, we tested the sera of this patient for antibodies to the MPER. We measured the neutralization activity of this serum against chimeric HIV-2 viruses engrafted with complete or partial HIV-1 MPER sequences (F. Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006). Using this method we were able to detect anti-MPER antibodies in serum from patient TM20 (Figure 7). This serum was able to neutralize viruses that carried the full MPER sequence of either subtype B (7312A-C1), C (7312A-C1C) or a more similar match to the autologous MPER sequence where positions 671 and 676 were mutated to serine (7312A-C1Cm). This neutralization response was mapped to the second half of the MPER as evidenced by the neutralization of C4, C4GW and C8, but not C3 or C7. It has previously been shown that the 4E10 MAb neutralizes the chimeras, C1, C4 and C6 at similar antibody concentrations (J. M. Decker et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006). Since TM20 sera failed to neutralize C6, this suggested the absence of 4E10–like antibodies. However, it partially neutralized the C4 chimera that in contrast to C6 includes the W680 residue which has been described to be important for 4E10 recognition (37). Taken together, our results suggest that the serum from this individual contained anti-MPER antibodies that recognize an epitope closely related, but not identical to the 4E10 epitope.

Neutralization of C1C F673L by serum from patient TM20: The mutation F673L observed in the clone TM20.13 was introduced in the HIV-2/HIV-1 chimeric virus C1C by site-directed mutagenesis (C1C F/L). This single mutation conferred resistance to the 4E10 MAb relative to the sensitive C1C parental virus (data not shown). The TM20 serum was

unable to neutralize the C1C F/L mutant virus (Figure 7), suggesting that the F673L mutation observed in the clone TM20.13 could indeed be an escape mutation from the anti-MPER response developed by this patient.

Discussion

In the present study we describe three viral quasispecies from an HIV-1 subtype C infected child with different sensitivities to neutralization by the broadly cross-reactive MAb 4E10. Neutralization resistance was conferred by a rare mutation F673L in the 4E10 epitope. In addition, moderate changes in sensitivity between clones were modulated by secondary positions in this epitope and motifs in the cytoplasmic tail. The presence of anti-MPER neutralizing antibodies in this individual supports the hypothesis that escape from antibody-mediated immune pressure was driving these changes.

The MAb 4E10 has been shown to be the most broadly cross-reactive antibody against HIV-1 (3), however it is not a particularly potent antibody, with >1 μg/ml needed to neutralize most viruses at 50%. In this study, the clone TM20.13 was resistant to neutralization at up to 100 μg/ml of 4E10 due to a mutation at F673L in the MPER. This is consistent with a report by Zwick and co-workers who showed that the introduction of the F673A mutation conferred resistance to 4E10 neutralization (37). The mutation F673L was present in a third of the quasispecies in this virus isolate. However, given that they were derived from an *in vitro* cultured virus, it is not clear if this mutation was present at higher or lower frequency *in vivo*, where neutralizing antibodies were present. While L673 is common amongst HIV-1 group O envelope sequences, this substitution is very rare among HIV-1 group M viruses in the Los Alamos HIV Sequence Database. The highly conserved nature of F673 suggests that this site either performs an important function or is not accessible to immune attack, as has been proposed (36). However, we found that virus infectivity was not obviously compromised by F673L (data not shown), similar to what was observed for the F673A mutant (37). This raises the question as to why this position is so highly conserved and under such strong negative selection pressure. Interestingly, in our study the F673L mutation was linked with the mutation S665K, which confers 2F5 sensitivity. All other clones were resistant to 2F5, due to the absence of a K at position 665, which is typical of subtype C viruses (3, 14). Furthermore, these mutations were accompanied in all cases by other mutations in gp41. HIV-1 is known to undergo considerable recombination during *in vivo* and *in vitro* proliferation (18), so the link between these changes may simply be the result of a recombination event between two divergent viral quasispecies. However, they could also represent compensatory changes associated with the mutation F673L that are required by the virus for survival *in vivo*. A

recent study on *in vitro* escape from 4E10 neutralization showed the appearance of F673L/V mutations in the resistant virus, but these viruses had impaired infectivity (19) which may explain why 4E10 escape variants were not observed in passive immunization studies with this antibody (22, 31). However, these viruses had no other genetic changes in this region, such as those shown here, that might have compensated for this loss of function (19).

TM20.5 and TM20.6 showed a 10-fold difference in sensitivity to 4E10. These clones were identical in the ectodomain of gp41 except for two positions (674 and 677) within the 4E10 binding domain. Previous studies have shown that these two residues are dispensable for 4E10 recognition (7, 37). However, we observed that the introduction of mutations N674D/N677K into TM20.5 increased 4E10 sensitivity by 3-fold, suggesting that these positions can influence the presentation of the 4E10 epitope. On the other hand, the TM20.6 clone did not become more resistant to 4E10 when these two positions were changed. Therefore, TM20.6 D674N/K677N which had an identical MPER to TM20.5 showed a 5-fold difference in neutralization sensitivity to 4E10. This was also noted in another study where viruses with identical 4E10 epitopes had different IC_{50} values (3) suggesting that factors outside the MPER affect accessibility of this epitope. Indeed, substitutions in the HR1 region of gp41 as well as other factors that affect fusion kinetics have been shown to influence sensitivity to this MAb (26, 37). Thus, the observation that TM20.5 showed enhanced infectivity compared to TM20.6 may explain why only TM20.5 was sensitive to changes at positions 674 and 677 in the MPER possibly as a result of a limited window of opportunity for neutralization.

We demonstrated that the cytoplasmic tail can modulate sensitivity to neutralization, in agreement with other studies (12, 16, 32, 34). Furthermore, we were able to identify four amino acids in the LLP-2 domain that affect both neutralization and infectivity. Such changes could impact on the amphipathicity of the LLP-2 α-helix and therefore its membrane association, resulting in phenotypic differences, as observed here and by others (16, 24). It has been shown that deletion of the cytoplasmic tail or changes in this region, in particular the LLP-2, determines the fusogenicity of the envelope glycoprotein (1, 17, 24, 33) which could result in changes in sensitivity to some entry inhibitors (1) and MAbs (16). Abrahamyan and co-workers suggested that the cytoplasmic tail hinders the folding

of the trimeric coiled-coil into the six-helical bundle resulting in greater inhibition by peptides that target the fusion intermediates (1). The fact that we did not observe changes in sensitivity to T-20, which also targets this conformation, suggests that the mutations in the LLP-2 did not influence this stage of the fusion process. On the other hand, early events may have been affected, such as the differential exposure of neutralizing epitopes in the native structure or, more likely, a faster kinetic rate from the CD4 bound conformation to the trimeric coiled-coil. This may preferentially impact 4E10 neutralization as this antibody is capable of binding in the post-attachment stage (2). In agreement with this, we found that 4E10 neutralization was more affected by changes in the LLP-2 than neutralization by IgG1b12 and CD4-IgG2.

Sensitivity to 4E10 neutralization was due to the combined effects of motifs in the MPER and the LLP-2. The two amino acid changes in the MPER and the four amino acid changes in the LLP-2 were sufficient to confer the 4E10 sensitive phenotype to TM20.5. However, engineering the more resistant phenotype appeared to require other sites in addition to these 6 amino acids. The fact that the TM20.6 MPER-5 LLP2-5 mutant showed the same sensitivity to 4E10 as the gp120(6)-gp41(5) construct, neither of which reached IC_{50} levels shown by TM20.5, suggested that perhaps sites in gp120 were also involved (Figure 5). Interestingly, the two mutations in the TM20.6 MPER, which failed to show any effect on their own, appeared to contribute to 4E10 resistance when co-expressed with the four LLP-2 mutations that increased infectivity; again highlighting the role that infectivity plays in determining overall neutralization sensitivity.

The presence of an anti-MPER neutralization response in this patient's serum supports the hypothesis that the observed 4E10 resistant envelope quasispecies constituted escape variants. Though the antibodies elicited against the MPER in this patient were not clearly 4E10-like as evidenced by the inability to neutralize C6, their epitopes overlapped, and as for 4E10, the residues F673 and W680 were important for recognition. A recent study with the antibody Z13e1 showed that the mutation D674N, as observed in TM20.5, eliminates antibody binding (23). Therefore, it is also possible that antibodies similar to Z13, where the amino acid at position 674 constitutes a critical residue, might be involved in this anti-MPER neutralization response.

This study strongly suggests that the MPER is indeed immunogenic and accessible to antibodies that can drive the evolution of the virus toward escape variants. However, such an argument is only demonstrable in the context of a longitudinal study where the evolution of changes in the envelope glycoprotein can be tracked over time and in parallel to the development of the neutralization response. Considering the interest in the MPER as a vaccine target, it is important that the potential of *in vivo* escape from anti-MPER antibodies be clarified in such longitudinal studies.

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Figure legends:

FIG 1: Frequency analysis of substitutions in the MPER region of 43 gp41 molecular clones obtained from the TM20 isolate. The substitutions K665S and F673L, associated with 2F5 and 4E10 resistance, respectively, are underlined and bolded. The functional envelope clones corresponding with some of these genotypes are indicated to the left of the sequences.

FIG 2: Neutralization of TM20 envelope clones. The three functional clones were tested for neutralization by (A) the MAb 4E10, 2F5 and the entry inhibitor T-20 (Enfuvirtide), and (B) polyclonal antibodies from two broadly cross-reactive HIV-1 positive plasma and autologous contemporaneous sera. The dotted lines indicate 50% neutralization with only those values above the line considered positive

FIG 3: Full length amino acid sequences of the functional envelope clones TM20.5, TM20.6 and TM20.13. Variable regions, heptad-repeat domains (HR-1 and HR-2), MPER, membrane spanning domain (MSD) and lentivirus lytic peptides (LLP-1 and LLP-2) are indicated. The mutation F673L in the MPER is underlined. The sensitivity of each of the clones to 2F5 and 4E10 neutralization is indicated with R denoting resistance and S denoting sensitivity. TM20.6 was extremely sensitive to 4E10 neutralization and is indicated with S++.

FIG 4: Changes in positions 674 and 677 in the MPER affect 4E10 neutralization. TM20.6, TM20.5 and mutants TM20.6 D674N/K677N and TM20.5 N674D/N677K were tested for their sensitivity to neutralization by 4E10. Neutralization was scored as the antibody concentration required to reduce infectivity by 50% (IC_{50}). The graph represents the mean of three independent experiments. P values are indicated when statistically significant differences between the means were observed in a Mann-Whitney nonparametric t-test analysis.

FIG 5: Changes in the cytoplasmic tail affect neutralization sensitivity. Schematic representation of the chimeras, constructed by exchanging gp120 or cytoplasmic tail segments of TM20.5 and TM20.6, and LLP-2 and MPER mutants. All the constructs were tested for 4E10, IgG1b12 and T-20 neutralization. The mean IC_{50} of three independent experiments are indicated on the right. The IC_{50} values of the chimeras and mutants were

compared to the parental clones TM20.5 or TM20.6 and the IC_{50} ratio shown in each case. Statistically significant differences between means of the parental and chimeric/mutant IC_{50} values with P values <0.05 and <0.01 in a Mann-Whitney t-test are highlighted in light and dark gray respectively.

FIG. 6: Role of gp120 and the cytoplasmic tail on infectivity and envelope incorporation. A) JC53bl-13 cells were infected with equal amounts of p24 (10 ng) of each parental and chimeric Env pseudotyped virus. Infectivity was determined by luciferase expression measured as Relative Light Units (RLU). The bars are colour coded according to the cytoplasmic tail carried by the construct, black for TM20.5 and white for TM20.6. B) Pelleted virions and C) *env* transfected cells, were lysed and subjected to SDS-PAGE, and visualized by Western blotting with anti-gp120 (D7312), anti-gp41 (7B2) or anti-p24 (D7312) antibodies. D) Schematic representation of the gp120, gp41 ectodomain and cytoplasmic tail encoded by the chimeric constructs. Regions derived from the clone TM20.5 are shaded grey and regions derived from TM20.6 are in white.

FIG 7: Anti-MPER neutralization activity present in TM20 serum. The HIV-1 MPER sequences introduced into the 7321A HIV-2 chimeric or mutant viruses used in the neutralization assay are highlighted in grey. The bolded letters represent the sequence of the intact 2F5 and 4E10 epitope. The mutations N671S and T676S in C1Cm and F673L in C1CF/L are underlined. The IC_{50} titers are indicated on the right with those showing activity highlighted in grey.

Figure 1

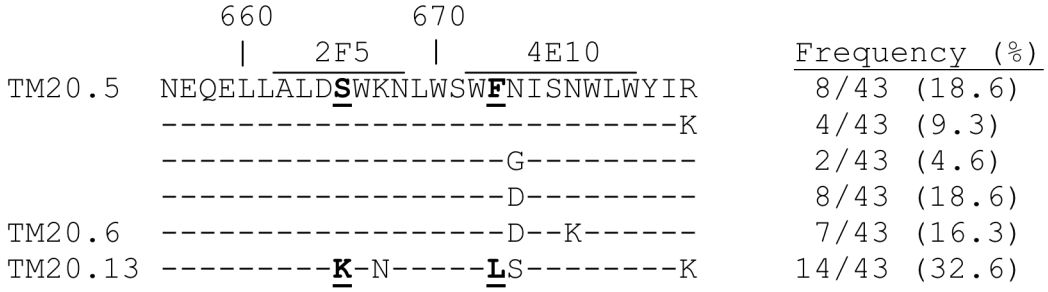

660
670
2F5
4E10
Frequency (%)
TM20.5 NEQELLALDSWKNLWSWFNISNWLWYIR 8/43 (18.6)
----------K 4/43 (9.3)
----------G--------- 2/43 (4.6)
----------D--------- 8/43 (18.6)
TM20.6 ----------D--K------ 7/43 (16.3)
TM20.13 ---------K-N-----LS--------K 14/43 (32.6)

Figure 2

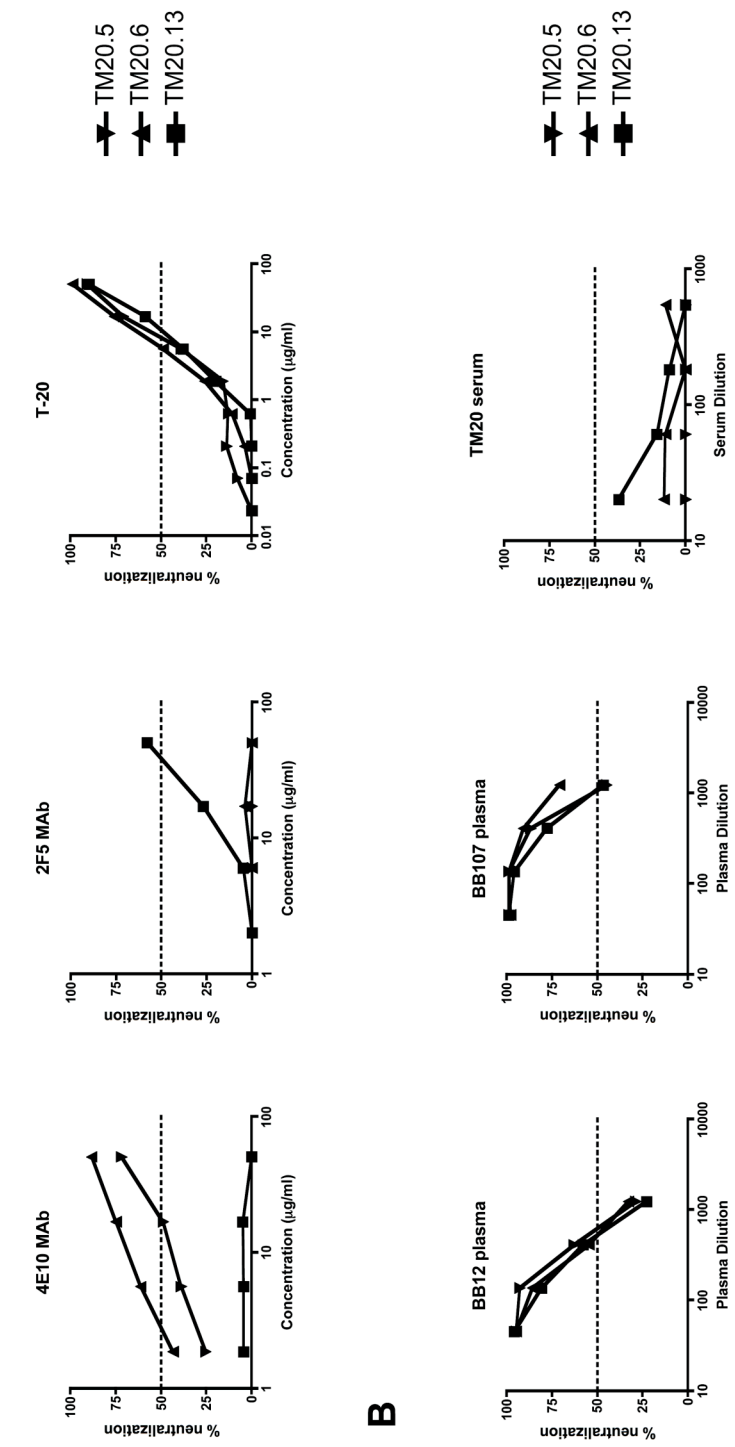

A
B
4E10 MAb
2F5 MAb
T-20
BB12 plasma
BB107 plasma
TM20 serum
% neutralization
Concentration (µg/ml)
Plasma Dilution
Serum Dilution
TM20.5
TM20.6
TM20.13

Figure 3

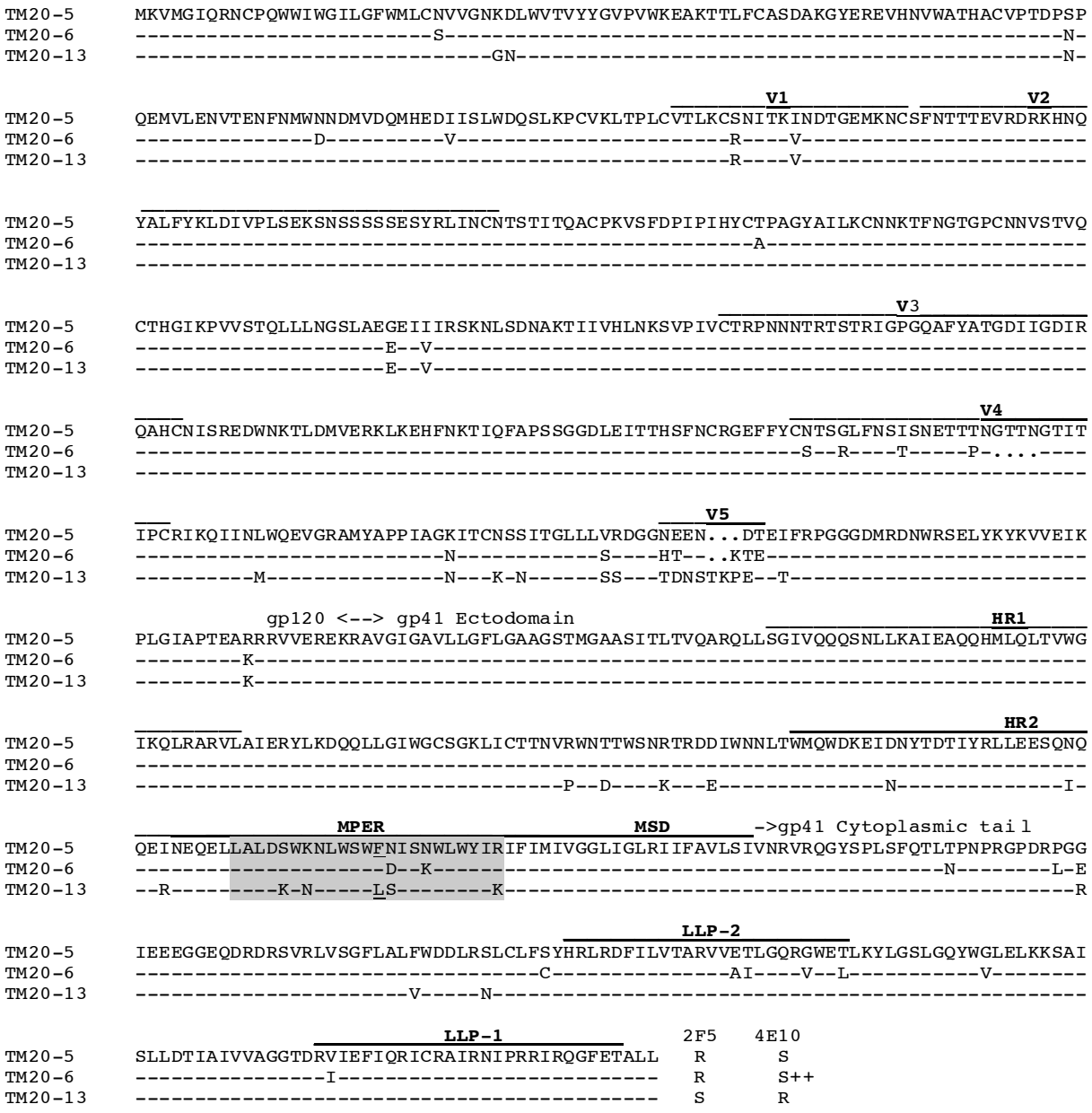

TM20-5 MKVMGIQRNCPQWWIWGILGFWMLCNVVGNKDLWVTVYYGVPVWKEAKTTLFCASDAKGYEREVHNVWATHACVPTDPSP
TM20-6 ----------S----------N-
TM20-13 ----------GN----------N-
V1
V2
TM20-5 QEMVLENVTENFNMWNNDMVDQMHEDIISLWDQSLKPCVKLTPLCVTLKCSNITKINDTGEMKNCSFNTTTEVRDRKHNQ
TM20-6 ----------D----------V----------R----V----------
TM20-13 ----------R----V----------
TM20-5 YALFYKLDIVPLSEKSNSSSSSESYRLINCNTSTITQACPKVSFDPIPIHYCTPAGYAILKCNNKTFNGTGPCNNVSTVQ
TM20-6 ----------A----------
TM20-13 ----------
V3
TM20-5 CTHGIKPVVSTQLLLNGSLAEGEIIIRSKNLSDNAKTIIVHLNKSVPIVCTRPNNNTRTSTRIGPGQAFYATGDIIGDIR
TM20-6 ----------E--V----------
TM20-13 ----------E--V----------
V4
TM20-5 QAHCNISREDWNKTLDMVERKLKEHFNKTIQFAPSSGGDLEITTHSFNCRGEFFYCNTSGLFNSISNETTTNGTTNGTIT
TM20-6 ----------S--R----T-----P-....----
TM20-13 ----------
V5
TM20-5 IPCRIKQIINLWQEVGRAMYAPPIAGKITCNSSITGLLLVRDGGNEEN...DTEIFRPGGGDMRDNWRSELYKYKVVEIK
TM20-6 ----------N----------S----HT--..KTE----------
TM20-13 ----------M----------N---K-N------SS---TDNSTKPE--T----------
gp120 <--> gp41 Ectodomain
HR1
TM20-5 PLGIAPTEARRRVVEREKRAVGIGAVLLGFLGAAGSTMGAASITLTVQARQLLSGIVQQQSNLLKAIEAQQHMLQLTVWG
TM20-6 ---------K----------
TM20-13 ---------K----------
HR2
TM20-5 IKQLRARVLAIERYLKDQQLLGIWGCSGKLICTTNVRWNTTWSNRTRDDIWNNLTWMQWDKEIDNYTDTIYRLLEESQNQ
TM20-6 ----------
TM20-13 ----------P--D----K---E----------N----------I-
MPER
MSD
->gp41 Cytoplasmic tail
TM20-5 QEINEQELLALDSWKNLWSWFNISNWLWYIRIFIMIVGGLIGLRIIFAVLSIVNRVRQGYSPLSFQTLTPNPRGPDRPGG
TM20-6 ----------D--K----------N--------L-E
TM20-13 --R----------K-N-----LS--------K----------R
LLP-2
TM20-5 IEEEGGEQDRDRSVRLVSGFLALFWDDLRSLCLFSYHRLRDFILVTARVVETLGQRGWETLKYLGSLGQYWGLELKKSAI
TM20-6 ----------C----------AI----V--L----------V--------
TM20-13 ----------V-----N----------
LLP-1
2F5 4E10
TM20-5 SLLDTIAIVVAGGTDRVIEFIQRICRAIRNIPRRIRQGFETALL R S
TM20-6 ----------I---------- R S++
TM20-13 ---------- S R

Figure 4

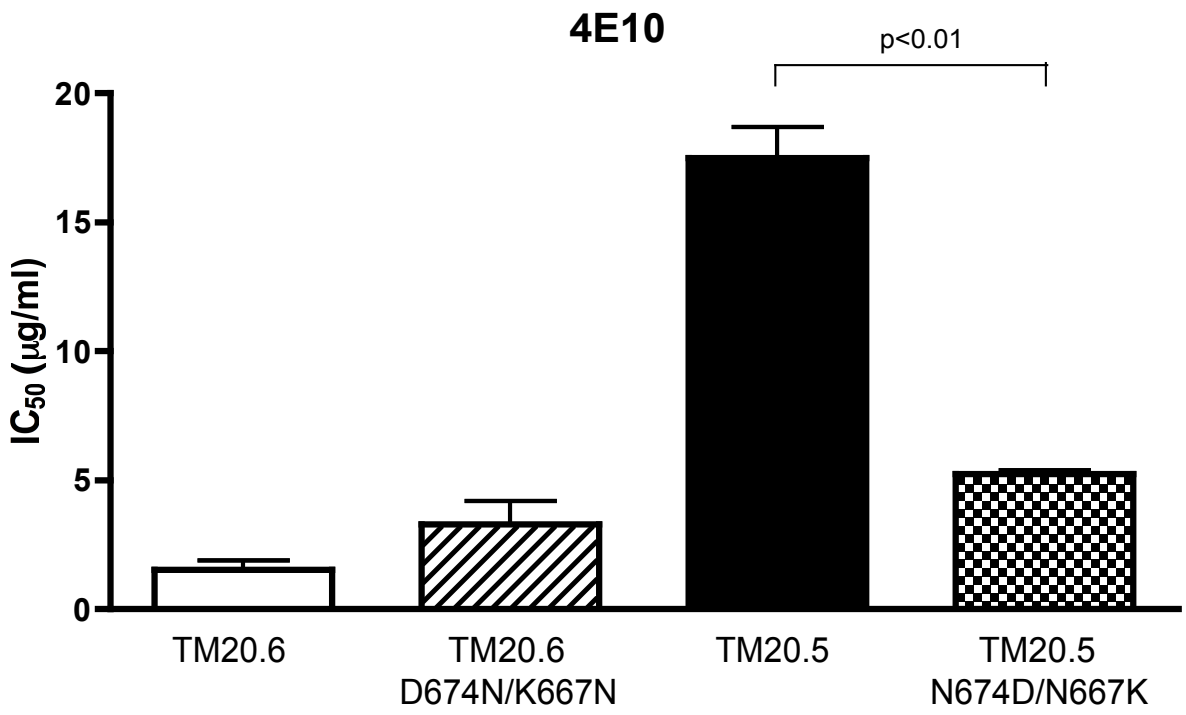
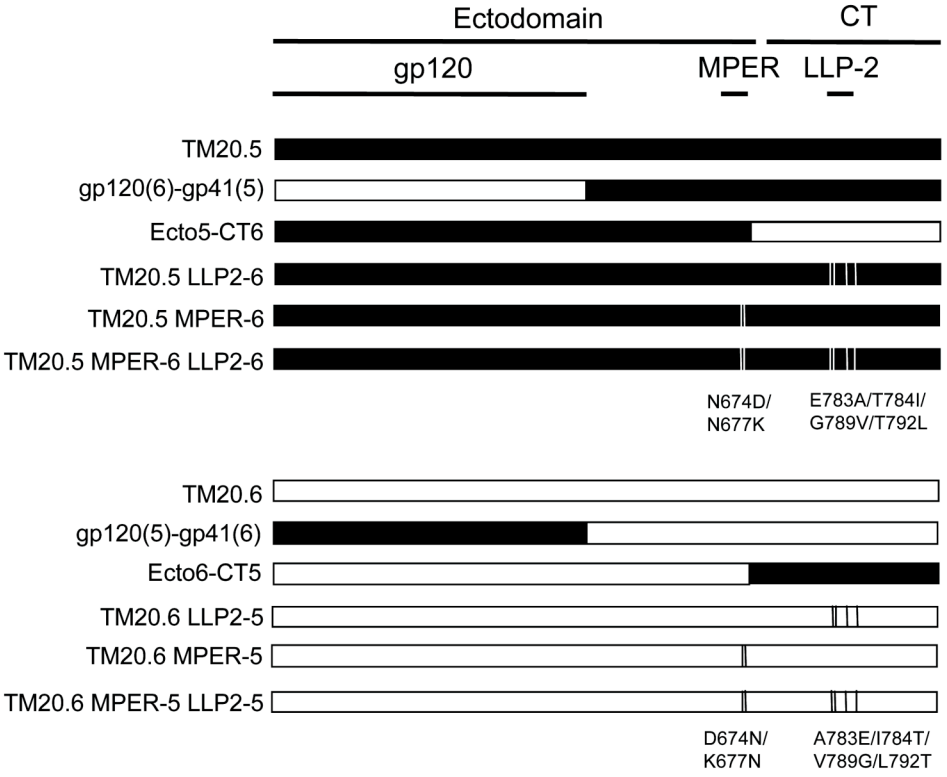

4E10
p<0.01
20
15
10
5
0
IC50 (μg/ml)
TM20.6
TM20.6 D674N/K667N
TM20.5
TM20.5 N674D/N667K

Figure 5



	Neutralization titers (µg/ml)					
	4E10		IgG1b12		T-20	
	IC50 ± STD	ratio	IC50 ± STD	ratio	IC50 ± STD	ratio
TM20.5	17.5 ± 2.0	1.0	7.0 ± 1.6	1.0	2.2 ± 0.2	1.0
gp120(6)-gp41(5)	12.1 ± 2.9	1.4	n/d		2.3 ± 0.1	1.0
Ecto5-CT6	4.6 ± 1.0	3.8	4.6 ± 0.6	1.5	1.7 ± 0.3	1.3
TM20.5 LLP2-6	3.7 ± 1.9	4.7	4.3 ± 1.5	1.6	1.9 ± 0.5	1.1
TM20.5 MPER-6	5.2 ± 0.3	3.3	6.7 ± 0.3	1.1	2.5 ± 0.3	0.9
TM20.5 MPER-6 LLP2-6	1.9 ± 0.8	9.1	3.4 ± 0.9	2.1	2.3 ± 0.2	1.0
TM20.6	1.5 ± 0.6	1.0	0.03 ± 0.01	1.0	2.3 ± 0.4	1.0
gp120(5)-gp41(6)	1.6 ± 0.7	1.0	n/d		1.8 ± 0.8	1.2
Ecto6-CT5	4.6 ± 1.5	3.0	0.05 ± 0.02	1.6	2.4 ± 0.5	1.0
TM20.6 LLP2-5	3.9 ± 0.8	2.6	0.05 ± 0.01	1.6	1.9 ± 0.1	0.8
TM20.6 MPER-5	3.3 ± 1.6	2.8	0.03 ± 0.01	0.9	1.9 ± 0.7	0.8
TM20.6 MPER-5 LLP2-5	11.3 ± 2.8	7.4	0.05 ± 0.01	1.6	2.0 ± 0.4	0.9

Figure 6

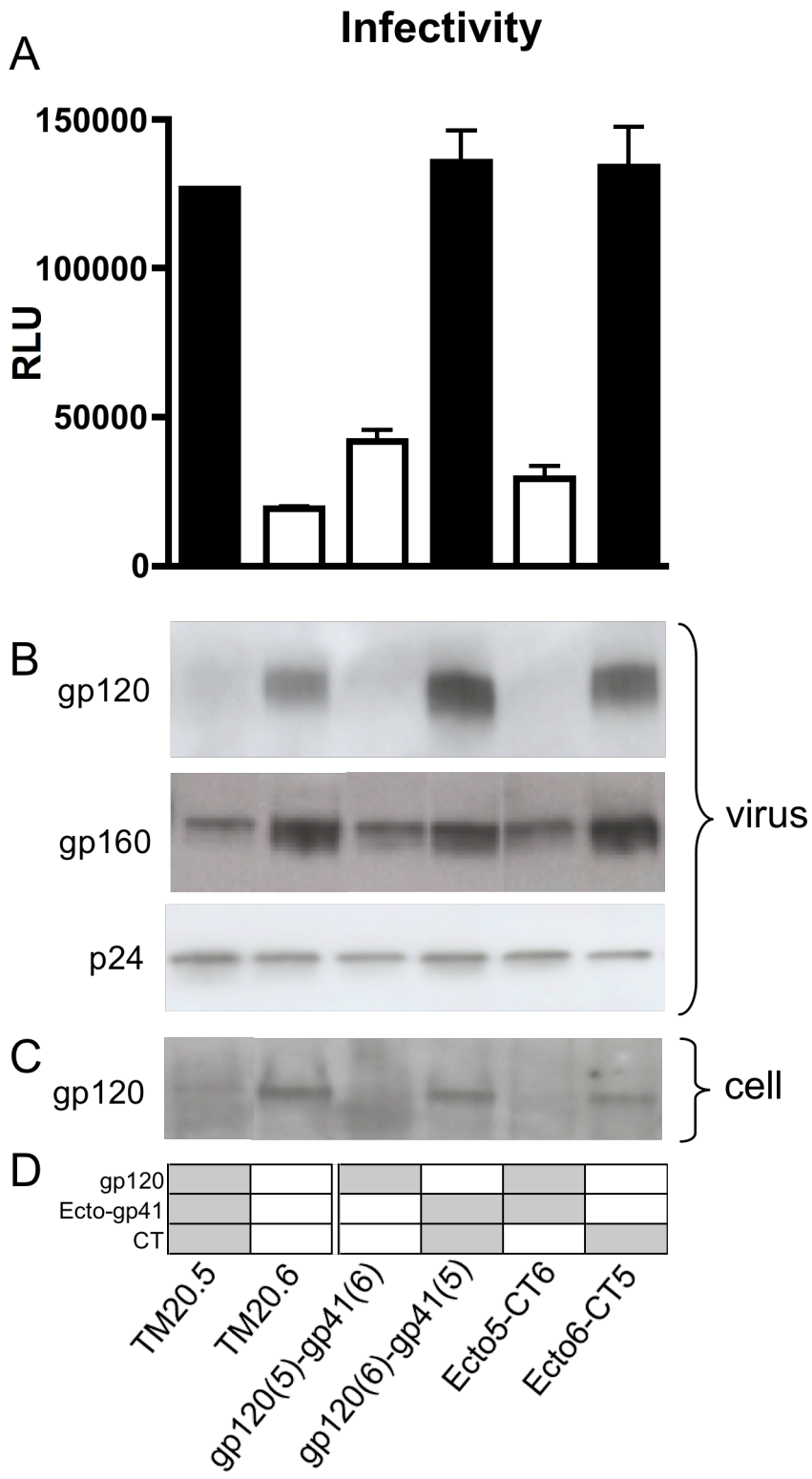
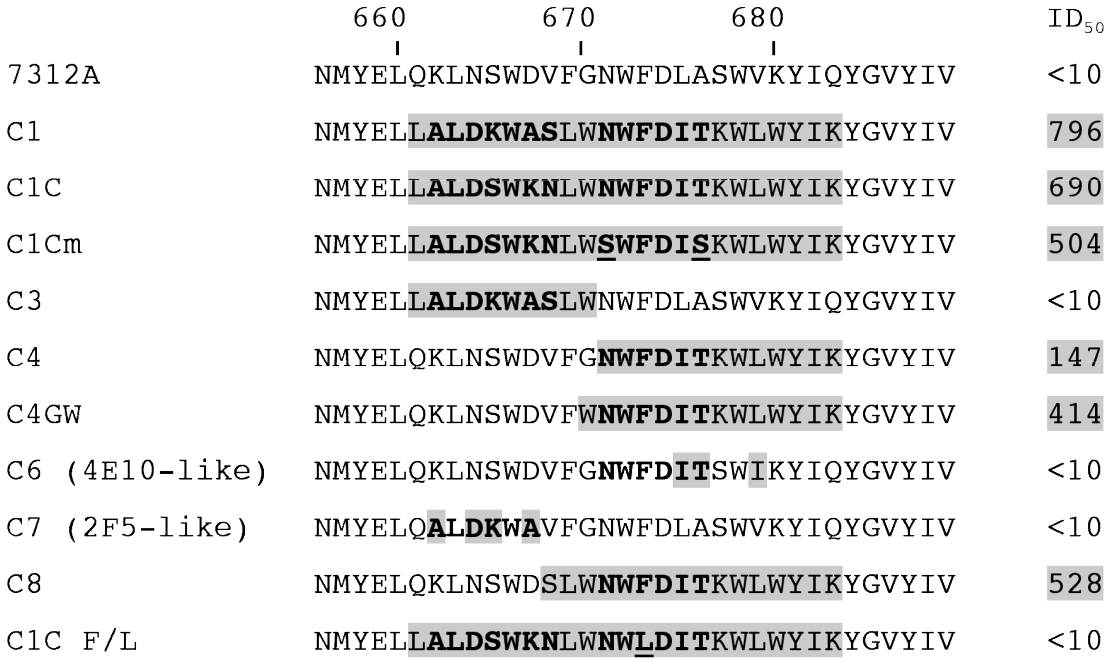

A
Infectivity
150000
100000
50000
0
RLU
B
gp120
gp160
p24
virus
C
gp120
cell
D
gp120
Ecto-gp41
CT
TM20.5
TM20.6
gp120(5)-gp41(6)
gp120(6)-gp41(5)
Ecto5-CT6
Ecto6-CT5

Figure 7


660
670
680
ID50
7312A NMYELQKLNSWDVFGNWFDLASWVKYIQYGVYIV <10
C1 NMYELLALDKWASLWNWFDITKWLWYIKYGVYIV 796
C1C NMYELLALDSWKNLWNWFDITKWLWYIKYGVYIV 690
C1Cm NMYELLALDSWKNLWSWFDISKWLWYIKYGVYIV 504
C3 NMYELLALDKWASLWNWFDLASWVKYIQYGVYIV <10
C4 NMYELQKLNSWDVFGNWFDITKWLWYIKYGVYIV 147
C4GW NMYELQKLNSWDVFWNWFDITKWLWYIKYGVYIV 414
C6 (4E10-like) NMYELQKLNSWDVFGNWFDITSWIKYIQYGVYIV <10
C7 (2F5-like) NMYELQALDKWAVFGNWFDLASWVKYIQYGVYIV <10
C8 NMYELQKLNSWDSLWNWFDITKWLWYIKYGVYIV 528
C1C F/L NMYELLALDSWKNLWNWLDITKWLWYIKYGVYIV <10

CHAPTER FIVE

NEUTRALIZING ANTIBODY RESPONSES IN ACUTE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C INFECTION

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Neutralizing Antibody Responses in Acute Human Immunodeficiency Virus Type 1 Subtype C Infection▽

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The study of the evolution and specificities of neutralizing antibodies during the course of human immunodeficiency virus type 1 (HIV-1) infection may be important in the discovery of possible targets for vaccine design. In this study, we assessed the autologous and heterologous neutralization responses of 14 HIV-1 subtype C-infected individuals, using envelope clones obtained within the first 2 months postinfection. Our data show that potent but relatively strain-specific neutralizing antibodies develop within 3 to 12 months of HIV-1 infection. The magnitude of this response was associated with shorter V1-to-V5 envelope lengths and fewer glycosylation sites, particularly in the V1-V2 region. Anti-MPER antibodies were detected in 4 of 14 individuals within a year of infection, while antibodies to CD4-induced (CD4i) epitopes developed to high titers in 12 participants, in most cases before the development of autologous neutralizing antibodies. However, neither anti-MPER nor anti-CD4i antibody specificity conferred neutralization breadth. These data provide insights into the kinetics, potency, breadth, and epitope specificity of neutralizing antibody responses in acute HIV-1 subtype C infection.

Neutralizing antibodies (NAbs) against the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein develop relatively slowly compared to HIV-specific CD8 T cells, which develop within weeks of infection (6, 16). This has led to the notion that antibody responses are less relevant to viral control, at least during the acute phase of infection. Recent technological advances in measuring NAb responses have shown that in some HIV-1-infected individuals, potent autologous responses can develop within a few months of infection, although others fail to develop such antibodies until much later (8, 13, 19). The fact that the envelope gene undergoes significant genetic variation which enables the virus to escape NAbs is testimony to the pressure exerted by these early autologous NAbs (19). Antibodies capable of neutralizing viruses other than the autologous virus take considerably longer to develop (8, 13), and only a few individuals develop truly broadly cross-reacting sera. These observations suggest that while there may be many targets for NAbs, few are located in highly conserved sites that might serve as suitable epitopes for inclusion in a vaccine immunogen.

The study of the antibody specificities of sera from HIV-1-infected individuals and of the relationship of those specificities to the breadth and potency of responses has become a topic of substantial interest, since this information may inform vaccine design (R. Wyatt, presented at the AIDS Vaccine 2005 International Conference, Montreal, Canada). Antibodies to CD4-induced (CD4i) epitopes are frequently found in HIV-1-infected individuals (1) and are thought to primarily target the coreceptor binding site, which includes the bridging sheet and, possibly, parts of the V3 region (20, 21). These polyclonal HIV-1-elicited antibodies, as well as a large number of different human monoclonal antibodies (MAbs) to HIV-1 CD4i epitopes (11, 21), can potently neutralize both HIV-1 and HIV-2 when they are pretreated with soluble CD4 (sCD4), indicating that the CD4i coreceptor binding surface is highly conserved antigenically among different subtypes of HIV-1 and the very divergent HIV-2 lineage (1).

Another site that has gained considerable attention recently as a target for NAbs is the membrane-proximal region (MPER), a linear stretch of 34 amino acids in gp41. MAbs targeting this region, such as 2F5 and 4E10, cross-neutralize a large fraction of HIV-1 isolates, and the MPER is therefore considered an important target for vaccines. However, antibodies with 2F5 or 4E10 binding specificity are rarely found in plasmas of HIV-1-infected individuals (23; J. M. Decker et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006), possibly due to their cross-reactivity with autoantigens, which results in clonal deletion of B cells with these specificities (4). The use of an HIV-2 chimeric envelope containing the HIV-1 MPER, however, has greatly facilitated our ability to study responses to epitopes throughout the MPER, and we have observed that approximately one-third of HIV-1-infected individuals develop such NAb responses (F. Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006).

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In this study, we explore the evolving NAb response over the first year of infection with HIV-1 subtype C. In addition to analyzing the autologous and heterologous NAb responses by conventional assays, we examined epitope-specific NAbs to CD4i and MPER epitopes in early infection in an effort to understand how such antibodies might contribute to neutralization breadth.

MATERIALS AND METHODS

A cohort of 245 high-risk, HIV-negative women was established in 2004 in Durban, South Africa, for follow-up and subsequent identification of HIV seroconversion. Detection of HIV infection was based on two HIV-1 rapid antibody tests (Determine [Abbott Laboratories, Tokyo, Japan] and Capillus [Trinity Biotech, Jamestown, NY]) performed monthly. Pooled PCR testing (Ampliscreen v1.5; Roche Diagnostics, Rotkreuz, Switzerland) for HIV-1 RNA was done on all antibody-negative samples. All positive samples identified through the pooling assay were confirmed using a quantitative RNA test and an HIV enzyme immunoassay (BEP 2000; Dade Behring, Marburg, Germany) on the same and subsequent samples. Women from this HIV-negative cohort, as well as other seroincidence cohorts, who had a reactive HIV antibody test within 3 months of a previously negative result or had detection by HIV-1 RNA PCR (Roche Amplicor v1.5) in the absence of HIV antibodies were enrolled in this study (CAPRISA 002). The timing of infection was determined as the midpoint between the last antibody-negative test and the first antibody-positive test or 14 days before the participant was PCR positive and antibody negative. Intense clinical follow-up and sample collection were done at enrollment, weekly for 3 weeks, fortnightly until 3 months, monthly until 12 months, and quarterly thereafter. CD4 T-cell counts were assessed using a FACSCalibur flow cytometer, and viral loads were measured using the COBAS AMPLICOR HIV-1 Monitor test, v1.5 (Roche Diagnostics). Plasmas, which were collected in EDTA, and sera were stored at −70°C until use. Written informed consent was obtained from all participants. This study received ethical approval from the University of the Witwatersrand, University of KwaZulu-Natal, and University of Cape Town.

Plasma samples, sCD4, and viruses. Plasma samples from HIV-1 subtype C-infected blood donors (BB8, BB12, BB28, BB55, BB70, and BB106) were purchased from the South African National Blood Service and previously described by Li et al. (9). Recombinant sCD4 was purchased from R&D Systems (Minneapolis, MN). The envelope clone SF162.LS was obtained from Leonidas Stamatatos (Seattle Biomedical Research Institute, Seattle, WA) (17). HIV-2 7312A (1) and derived chimeras were obtained from George Shaw (University of Alabama, Birmingham, AL).

Cell lines. The JC53-bl cell line, engineered by John Kappes and Xiaoyun Wu, was obtained from the NIH AIDS Research & Reference Reagent Program. 293T cells used for transfection were obtained from George Shaw (University of Alabama, Birmingham, AL) and David Montefiori (Duke University School of Medicine, Durham, NC). Both cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies) containing 10% heat-inactivated fetal bovine serum and 50 μg/ml gentamicin (Sigma). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA.

Cloning of envelope genes. Long-template HIV-1 cDNA transcripts were generated from viral RNAs extracted from plasma. Whole genomes were amplified from cDNA by using a modified limiting dilution nested PCR assay as described by Rousseau et al. (14). First-round whole-genome products were used as templates to amplify full-length envelope genes. The 3-kb PCR fragments, generated using the envA and envM primers (2), were cloned into the pCDNA 3.1-TOPO vector (Invitrogen) and screened as previously described (3). Env-pseudotyped viruses were obtained by cotransfecting the Env plasmid with pSG3deltaEnv (19), using Fugene transfection reagent (Roche).

Neutralization assay. Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of JC53-bl cells with Env-pseudotyped viruses (10). Titers were calculated as the inhibitor concentrations (IC_{50}) or reciprocal plasma/serum dilutions (ID_{50}) causing a 50% reduction of relative light units.

CD4i and MPER neutralization assays. CD4i and MPER neutralization assays were performed as described by Decker et al. (1), using the HIV-2 virus 7312A and the HIV-2/HIV-1 MPER chimeras described in Fig. 9. Briefly, 2,000 IU of virus was incubated with fivefold dilutions of plasma/serum (starting dilution, 1:20). After 1 hour, the mixture was added to 40% confluent JC53-bl cells, which had been seeded the day before in a 96-well plate. Infection was measured 48 h later by evaluating the luciferase activity. To evaluate the CD4i antibody response, the virus was preincubated for 1 h with sCD4 at a concentration equal to the IC_{50} for each virus strain before adding the diluted plasma/serum.

gp160 sequencing. Cloned *env* genes were sequenced using an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and then resolved on an ABI 3100 automated genetic analyzer. The full-length gp160 sequences were assembled and edited using Sequencher v. 4.0 software (Genecodes, Ann Arbor, MI). The number of potential N-linked glycosylation sites (PNGS) was determined using the software N-glycosite (http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html). Multiple alignments were performed using Clustal X (version 1.83) and were edited with BioEdit (version 5.0.9). Phylogenetic analysis was performed with MEGA, version 2.1. A neighbor-joining tree was constructed with distances calculated using Kimura's two-parameter model, with reference sequences obtained from the Los Alamos Sequence Database (http://hiv-web.lanl.gov). Bootstrap values are the result of 1,000 resamplings.

Statistical analysis. Correlation analysis was performed by using Graphpad Prism 4.0 software to perform Spearman's nonparametric rank test. Correlations were considered statistically significant when P values were ≤ 0.05.

Nucleotide sequence accession numbers. The GenBank database accession numbers for the *env* clones described in this study are EF203957 to EF203989.

RESULTS

Development of autologous NAb response against early virus. Fourteen female sex workers with acute HIV-1 infection were identified prospectively as part of the CAPRISA 002 study. These women were estimated to have been infected for a median of 5 weeks (range, 2 to 8 weeks) at the time of enrollment (Table 1). Plasma RNA from the enrollment sample was used to amplify the envelope gene for each participant. The resultant *env* amplicons were cloned into an expression vector and cotransfected with a subtype B backbone to generate Env-pseudotyped viruses. Sequence analysis indicated that these envelope clones grouped together with the population sequence from each individual, and all sequences clustered significantly with HIV-1 subtype C reference sequences (data not shown).

The development of the autologous NAb response was examined, using one to three clones per participant and serum samples collected at roughly bimonthly intervals over 12 months (Fig. 1). The neutralization sensitivities of the multiple clones derived from each sample were very similar, and analysis of the sequences showed that there was minimal genetic diversity at this time point (data not shown). The Env-pseudotyped viruses were examined for coreceptor usage, using GHOST-3 cells expressing CD4 and either CCR5 or CXCR4. All Env-pseudotyped viruses used the CCR5 coreceptor, not the CXCR4 coreceptor. Examination of the V3 sequences using the subtype C position-specific scoring matrix (5) also predicted CCR5 usage.

Analysis of the autologous NAb response against the early envelopes showed a large degree of variation between participants in the kinetics and magnitude of the response (Fig. 1). Most women showed an increase in titer over the first 6 months, with a median time to first detection of 19 weeks, with titers reaching a plateau at between 6 and 12 months. Eight women developed potent NAb responses, with titers of >1:1,000, by 12 months, while two, CAP61 and CAP210, developed weak responses within 12 months of infection. One of these (CAP210) was a rapid progressor, while the other (CAP61) was a controller (Table 1). There was no correlation between the patient's clinical status and the magnitude or kinetics of the autologous NAb response.

TABLE 1. Clinical data for 14 acutely HIV-1 subtype C-infected individuals

Patient no.[a]	Date of blood draw	Time (wk) postinfection	Viral load (RNA copies/ml)	CD4 count (cells/μl)	Clinical status[b]
002-10-0008	17 May 2005	3	373,000	360	
002-10-0045	11 May 2005	5	236,000	974	Controller
002-10-0061*	20 December 2004	8	610	389	Controller
002-10-0063	19 January 2005	4	277,000	414	Rapid progressor
002-10-0084	28 February 2005	3	9,140	636	
002-10-0085	22 June 2005	5	621,000	419	
002-10-0088*	17 February 2005	5	29,400	963	
002-10-0206	12 July 2005	8	368,000	365	Rapid progressor
002-10-0210	25 May 2005	5	127,000	461	Rapid progressor
002-10-0228	11 May 2005	7	3,840	867	Controller
002-10-0239	10 August 2005	5	95,800	845	
002-10-0244*	23 May 2005	8	19,200	557	
002-10-0255*	21 June 2005	8	196,000	693	
002-10-0256	5 September 2005	6	56,500	689	

[a] *, patients from the seroincidence cohort. The rest of the patients were from the sex worker cohort.
[b] Controller, individual with a CD4 count of >350 cells/ml and a viral load of <2,000 copies/ml on at least two consecutive measurements after 6 months of infection; rapid progressor, individual with a CD4 count of <350 cells/ml and a viral load of >100,000 copies/ml on at least two consecutive measurements after 6 months of infection.

Envelope sensitivity to autologous neutralization depends on length and N-linked glycosylation of the variable loops. To assess whether the neutralization titers correlated with the genetic characteristics of the envelope gene, we analyzed the length of variable loops and the number of PNGS. The length of the V1-to-V5 region, as well as the number of PNGS in this region, correlated inversely with autologous NAb titers at 12 months postinfection (Fig. 2a and d). The correlation of titer

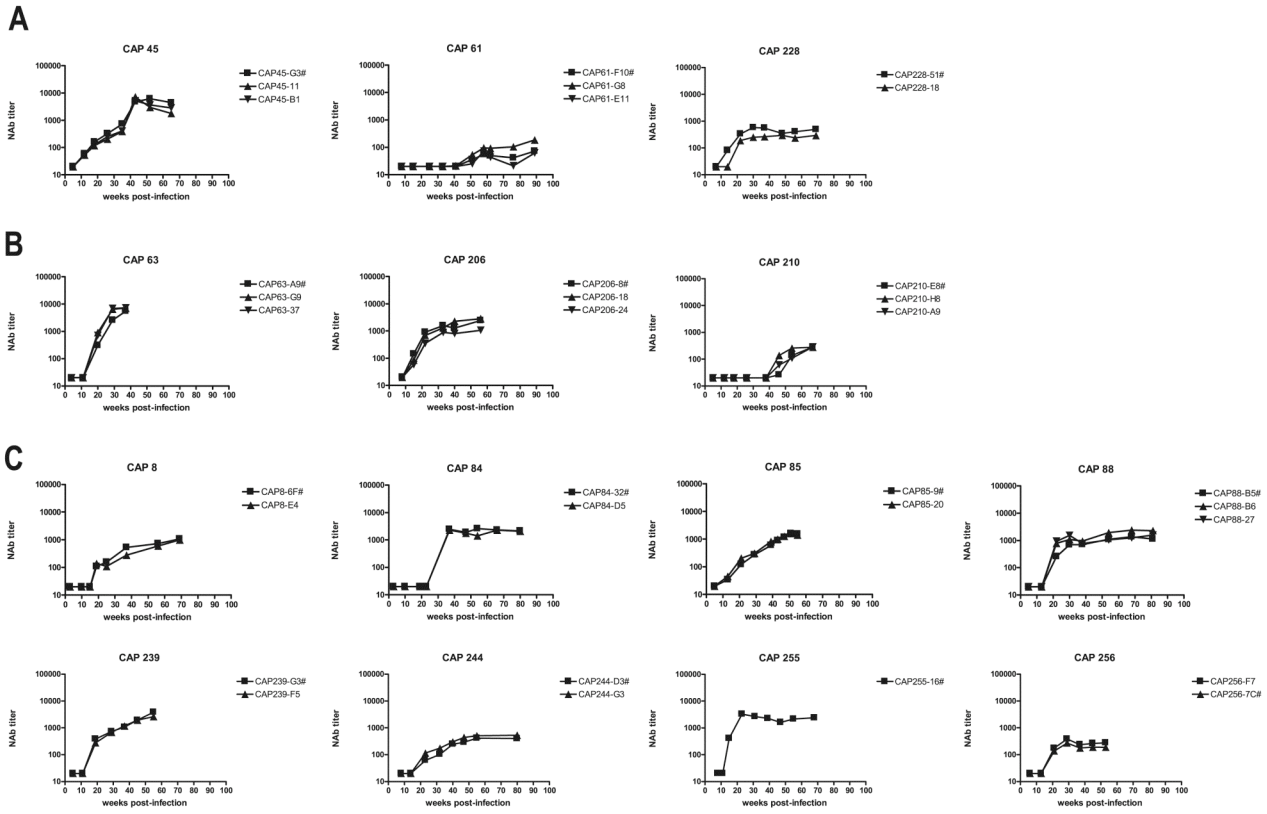

FIG. 1. Comparison of autologous neutralization sensitivities of multiple envelope clones from individual patients. One to three functional envelope clones at a single time point (as indicated in Table 1) for each of 14 acutely infected patients were tested against autologous serum in an Env-pseudotyped virus neutralization assay. Results are shown as IC_{50} values (neutralization titers) over time among participants defined as controllers (A), rapid progressors (B), or normal progressors (C) according to criteria listed in Table 1. The clones marked with a pound sign were used in all subsequent experiments.

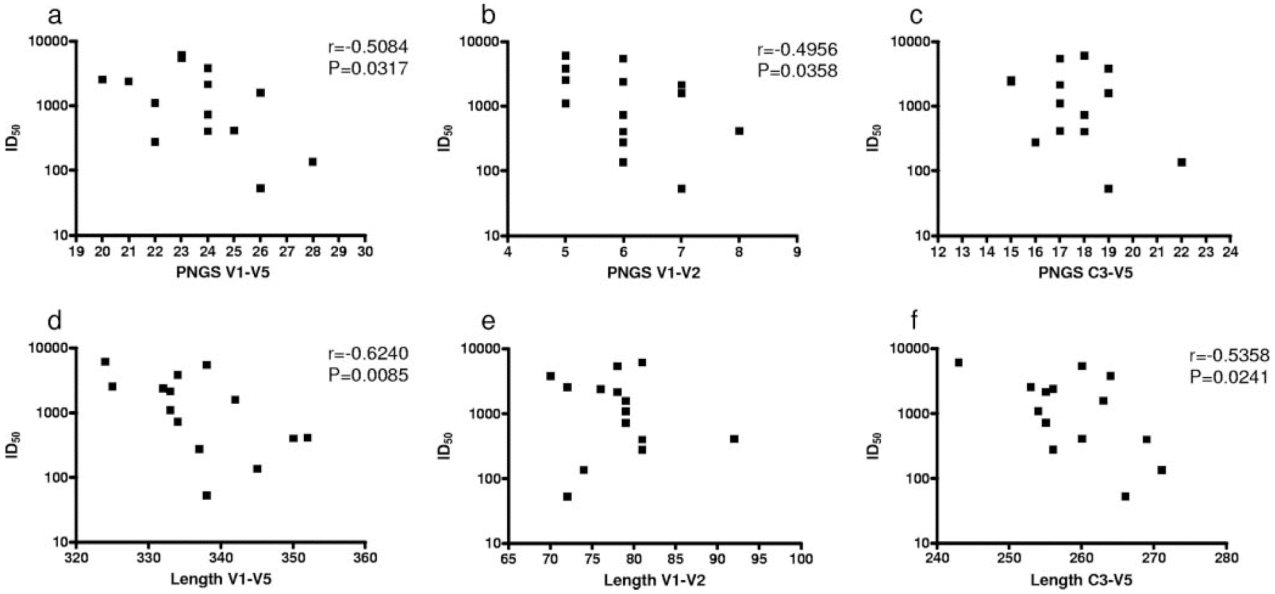

FIG. 2. Correlation between number of N-linked glycosylation sites, variable loop length, and autologous neutralization titer. The autologous neutralization titer at 12 months of infection for each of the 14 study subjects was plotted against the numbers of PNGS in the V1-to-V5 (a), V1-V2 (b), and C3-to-V5 (c) regions or the lengths of the V1-to-V5 (d), V1-V2 (e), and C3-to-V5 (f) regions of the matched viral clone. The Spearman *r* coefficient and the *P* value are shown when the correlation is significant ($P < 0.05$).

with V1-to-V5-region length ($P = 0.0085$) was stronger than that with the number of PNGS ($P = 0.0317$). Analysis of the V1-V2 and C3-to-V5 regions independently demonstrated that the number of PNGS in the V1-V2 loop, but not the length of the V1-V2 loop, was associated with resistance to autologous neutralization (Fig. 2b and e).

Early NAbs are isolate specific. The potency of the early autologous responses prompted us to examine the ability of the

6th months serum	Enrolment virus													
	CAP 8	CAP 45	CAP 61	CAP 63	CAP 84	CAP 85	CAP 88	CAP 206	CAP 210	CAP 228	CAP 239	CAP 244	CAP 255	CAP 256
CAP 8	**155**	<20	**34**	<20	<20	**136**	<20	**37**	<20	<20	<20	**29**	<20	<20
CAP 45	<20	**327**	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CAP 61	<20	<20	<20	<20	**27**	**66**	<20	<20	<20	<20	<20	<20	<20	<20
CAP 63	<20	<20	<20	**2572**	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CAP 84	**22**	<20	<20	<20	**2443**	**21**	<20	<20	<20	<20	<20	<20	<20	<20
CAP 85	<20	<20	<20	<20	<20	**273**	<20	<20	<20	<20	<20	<20	<20	<20
CAP 88	**27**	<20	<20	<20	<20	<20	**257**	<20	<20	<20	<20	<20	<20	<20
CAP 206	<20	<20	<20	<20	<20	**82**	<20	**1577**	<20	<20	<20	<20	<20	<20
CAP 210	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CAP 228	<20	<20	**29**	<20	**27**	**36**	<20	<20	<20	**576**	<20	<20	<20	<20
CAP 239	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	**731**	<20	<20	<20
CAP 244	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	**103**	<20	<20
CAP 255	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	**2668**	<20
CAP 256	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	**272**

12th months serum	Enrolment virus													
	CAP 8	CAP 45	CAP 61	CAP 63	CAP 84	CAP 85	CAP 88	CAP 206	CAP 210	CAP 228	CAP 239	CAP 244	CAP 255	CAP 256
CAP 8	**733**	<20	**40**	<20	**29**	**248**	<20	**37**	<20	<20	<20	**29**	**23**	<20
CAP 45	<20	**6199**	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CAP 61	<20	<20	**54**	<20	**145**	**84**	<20	<20	<20	<20	**32**	<20	<20	<20
CAP 63	<20	<20	<20	**5490**	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
CAP 84	<20	<20	<20	<20	**2585**	**21**	<20	<20	<20	<20	<20	<20	<20	<20
CAP 85	**29**	<20	<20	**113**	<20	**1697**	<20	<20	<20	<20	<20	<20	<20	<20
CAP 88	<20	<20	<20	<20	<20	**33**	**1097**	<20	<20	<20	<20	<20	<20	<20
CAP 206	<20	<20	<20	<20	<20	**236**	<20	**2425**	<20	<20	<20	<20	<20	<20
CAP 210	<20	<20	<20	<20	<20	<20	<20	<20	**138**	<20	<20	<20	<20	<20
CAP 228	<20	**36**	<20	<20	**27**	**41**	<20	**51**	<20	**405**	<20	<20	<20	<20
CAP 239	<20	<20	**37**	<20	**28**	<20	<20	<20	<20	<20	**3841**	<20	<20	<20
CAP 244	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	**416**	<20	<20
CAP 255	<20	<20	**96**	<20	<20	**78**	<20	<20	<20	<20	**67**	<20	**2163**	<20
CAP 256	<20	**195**	**27**	<20	<20	<20	<20	<20	<20	<20	**85**	<20	<20	**188**

FIG. 3. Heterologous neutralization of enrollment viruses by sera obtained at 6 and 12 months postinfection. Sera obtained at 6 and 12 months postinfection from all 14 patients were tested for neutralization against a representative Env-pseudotyped virus clone from each patient. The reciprocal ID_{50} values are shown, and those in bold show where neutralization was observed. The highlighted (boxed) cells represent autologous neutralization.

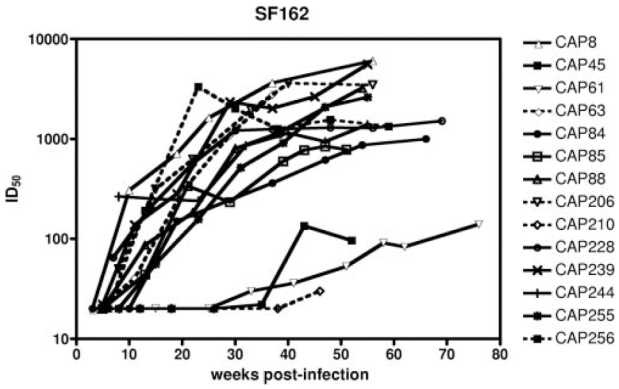

FIG. 4. Neutralization of SF162, a neutralization-sensitive subtype B virus. Sequential serum samples from 14 HIV-1 subtype C-infected patients were tested for neutralizing activity against SF162. Results are shown as the ID_{50} values (neutralization titers) over time.

sera to cross-neutralize other envelopes within the CAPRISA cohort. Sera collected at 6 months and 12 months postinfection showed very little heterologous neutralization (Fig. 3). Thus, while the matched serum-envelope combinations showed high titers, this was not the case for nonmatched serum-envelope combinations. We did observe a slight increase in breadth at 12 months postinfection, which suggests that the capacity for cross-neutralization will likely increase over time. Of particular interest was patient CAP61, whose serum neutralized an unmatched envelope (from CAP84) better than the autologous envelope.

Despite the absence of cross-neutralizing antibodies for primary viruses in these women, most of them were able to neutralize the highly sensitive subtype B virus SF162 (Fig. 4). However, three participants, two of whom also had low autologous NAbs (CAP61 and CAP210), had much lower titers against SF162 than did the rest of the cohort. In contrast, although patient CAP45 developed a strong autologous NAb response (Fig. 1A), this individual failed to neutralize SF162 or any other of the viruses tested, suggesting a highly type-specific neutralization response.

Sensitivity of early HIV-1 subtype C envelopes to antibody neutralization. The neutralization sensitivities of the envelope clones used in this study were tested using six broadly cross-reactive subtype C plasma samples (Fig. 5). Most envelopes were relatively resistant to neutralization, similar to the subtype C reference panel (9) and as expected for primary viruses. There was no correlation between the sensitivity to heterologous neutralization and clinical status. The most sensitive envelopes were those of the CAP84 and CAP85 clones (geometric mean titer [GMT], 489 and 780, respectively). In addition to having an unusually high GMT when tested against subtype C plasma, the CAP85 clone showed the highest sensitivity to neutralization by heterologous CAPRISA sera (Fig. 3). This suggests a neutralization-sensitive phenotype for this envelope clone. However, unlike many neutralization-sensitive viruses, it was not sensitive to sCD4 (IC_{50}, 15.5 μg/ml) (data not shown). The clones most resistant to heterologous neutralization were those from patients CAP8 and CAP256 (GMT, 101 and 82, respectively). The two clones that were relatively resistant to neutralization by the autologous sera (from patients CAP61 and CAP210) displayed a mid-range GMT value. This suggests that the failure of autologous sera to neutralize these viruses is not simply the result of an envelope with a neutralization-resistant phenotype.

We evaluated whether the lengths of the variable regions and the number of PNGS correlated with the sensitivity to heterologous neutralization of each envelope clone, excluding the CAP85 clone. Overall sensitivity, as assessed by the number of CAPRISA sera able to neutralize each clone, was correlated with the length of the V1-V2 region (Fig. 6A). Furthermore, the GMT for a heterologous panel of subtype C-infected plasmas also correlated significantly with V1-V2 length (Fig. 6B). Correlations of heterologous neutralization sensitivity with the length of the C2-to-V5 region and the number of PNGS were not significant (data not shown).

Antibodies to CD4i epitopes. Antibodies to CD4i epitopes were measured using an HIV-2 envelope, as described by Decker et al. (1). We found that 12 of 14 HIV-1 subtype C-infected participants developed CD4i antibodies and that these antibodies were generally characterized by variable kinetics and their early appearance following infection (Fig. 7). We observed a weak correlation between the magnitude of the CD4i antibody response and the number of heterologous viruses neutralized ($P = 0.04$). Interestingly, three women, including two rapid progressors, had high titers of CD4i antibodies at the earliest time tested. Earlier, preinfection samples

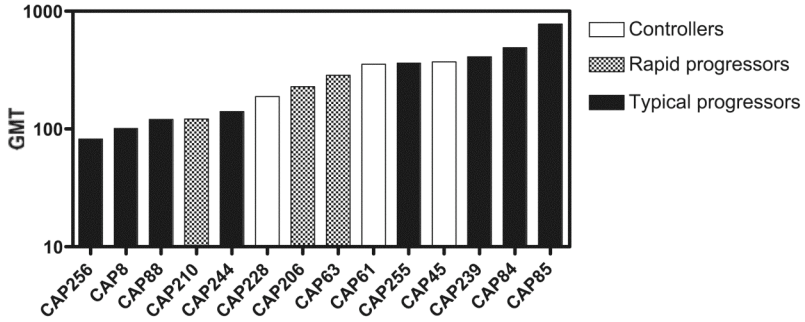

FIG. 5. Neutralization sensitivities of early envelope clones from HIV-1 subtype C-infected patients. The neutralization sensitivities of 14 Env-pseudotyped viruses are represented as the GMTs for neutralization by six HIV-1-positive plasma samples (BB8, BB12, BB28, BB55, BB70, and BB106). The bars indicate the clinical status of the patients from whom the clones were derived.

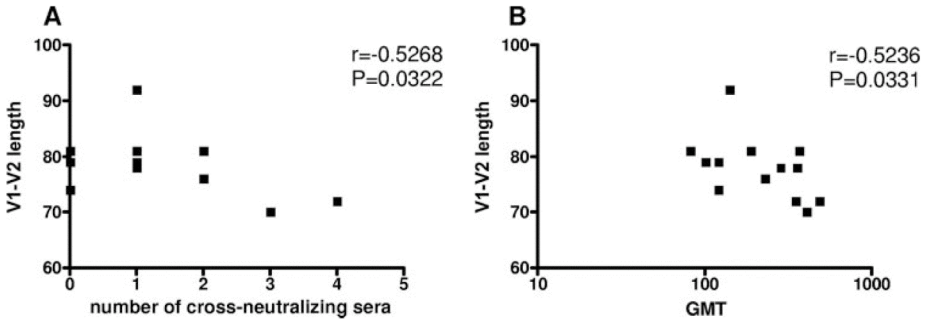

FIG. 6. Correlation between neutralization sensitivity and genetic characteristics of the envelope. The length of the V1-V2 region of each envelope clone tested for heterologous neutralization was plotted against the number of cross-neutralizing sera (derived from Fig. 3) or the GMT of six HIV-1-positive plasma samples (Fig. 5). The Spearman *r* coefficient and the *P* value are depicted for each correlation.

collected from two of these women were negative for CD4i antibodies, confirming that these antibodies were indeed induced by HIV-1 infection (Table 2). The appearance of these CD4i antibodies before the autologous strain-specific NAb response gave us the opportunity to test the capacity of these Abs to neutralize the autologous virus in the presence of sCD4. No neutralization was detected when the autologous virus was preincubated with sCD4 at its IC_{50} (Table 2), which is consistent with earlier work where we found the HIV-1 CD4i coreceptor binding surface to be less accessible than that on HIV-2, even in the presence of sCD4 (1).

Antibodies to gp41 MPER epitopes. Anti-MPER antibodies were measured using the HIV-2 7312A envelope bearing a subtype C MPER, referred to as C1C. Two of the 14 women (CAP85 and CAP206) developed high titers of anti-MPER antibodies within 6 months of infection (peaking at 40 weeks postinfection and reaching titers of approximately 1:1,000), with three additional women developing lower titers later in infection (Fig. 8). In order to map more precisely the region within the MPER targeted by these antibodies, sera from CAP85 and CAP206 were tested against additional chimeric mutants that carry small regions or only point mutations of the MPER, as shown in Fig. 9. Two specific constructs, 7312A-C3 and 7312A-C6, allow the detection of 2F5- and 4E10-like antibodies, respectively (Decker et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006). For both CAP85 and CAP206, sera contained antibodies to the 7312A-C1, 7312A-C8, and 7312A-C4GW chimeras, suggesting that these anti-MPER NAbs target the C-terminal region of the MPER. Interestingly, the lack of neutralization of the 7312A-C4 chimera indicated that W670 is important for recognition. While these antibodies recognized a region that overlaps the 4E10 epitope, they were not 4E10-like because they failed to neutralize the 7312A-C6 construct (Bibollet-Ruche et al., presented at the Keystone Symposium on HIV Vaccines, Keystone Resort, Keystone, CO, 2006).

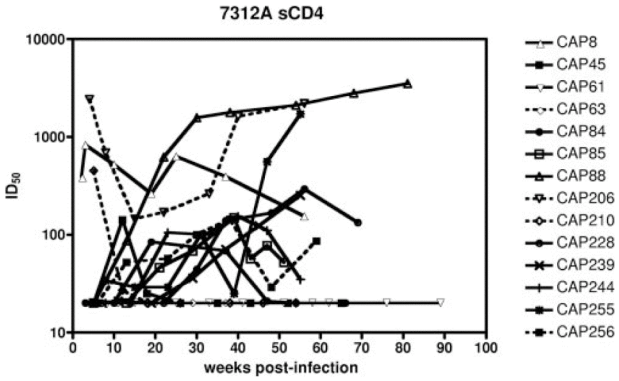

FIG. 7. CD4i NAb responses in acute HIV-1 subtype C infection. Sera were assayed using an HIV-2 (7312A) envelope in the presence of 9 nM sCD4. Titers are shown as ID_{50} values over time. No neutralization was observed in the absence of sCD4 (data not shown).

DISCUSSION

In this study, we report that individuals infected with HIV-1 subtype C developed a potent autologous NAb response between 3 and 12 months after infection, with a median time of 19 weeks. However, these antibodies were highly type specific and rarely neutralized heterologous viruses from the same cohort. Antibodies to CD4i epitopes were present in most participants, often earlier than the autologous antibodies, and in some within weeks of infection. NAbs to the MPER developed much later and in fewer individuals. Collectively, these data provide important insights into the early autologous antibody responses in HIV-1 subtype C infection.

Previous studies of HIV-1 subtype B-infected individuals have shown that autologous NAbs develop within months of infection (13, 19). While the times to peak titer were shown to be similar for a subtype C-infected cohort, with a range of 7 to 24 months, titers were, on average, 3.5-fold higher (8). We also report high titers in this subtype C-infected cohort, with 8 of 14 women reaching titers in excess of 1:1,000 within the first year of infection, though in some cases they had not yet peaked. In a study by Li and coworkers, an analysis of the genetic characteristics of recently transmitted viruses showed that subtype C envelopes were more compact, with shorter V1-to-V4 regions, than their subtype B counterparts and that this inversely correlated with the magnitude of the autologous neutralization response (8). Thus, the higher titers of autologous antibodies in subtype C infection may be more related to a generally neutralization-sensitive phenotype than with higher antibody titers per se (9). In this study, we also found that the length of

TABLE 2. Detection of CD4i antibodies in two patients prior to and after HIV-1 infection[a]

Patient no.	Date of blood draw	HIV-1 enzyme-linked immunosorbent assay result	Pooled RNA PCR result	Time (wk) postinfection	Viral load (copies/ml)	CD4 T-cell count (cells/μl)	Autologous neutralization[b]		Neutralization of 7312A[b]	
							Without sCD4	With sCD4	Without sCD4	With sCD4
002-10-0008	28 September 2004	Negative	Negative	−30	NA	756	ND		20	20
	6 January 2005	Negative	Negative	−16	NA	ND	ND		20	20
	3 March 2005	Negative	Negative	−8	NA	ND	ND		20	20
	11 May 2005	Positive	ND	2	207,000	ND	20	20	20	393
	17 May 2005	Positive		3	373,000	360	20	20	20	823
	6 July 2005			10	368,000	467	20		20	521
	7 September 2005			19	73,100	329	108		20	263
	19 October 2005			25	98,400	343	90		20	631
	11 January 2006			37	27,600	227	176		20	392
	23 May 2006			56	39,300	332	619		20	154
002-10-0206	21 April 2005	Negative	Negative	−4	NA	1,643	ND		20	20
	15 June 2005	Positive	ND	4	196,000	ND	20	20	20	2,429
	12 July 2005			8	368,000	365	20	20	20	687
	30 August 2005			15	113,000	292	145		20	145
	20 October 2005			22	127,000	ND	906		20	170
	3 January 2006			33	138,000	325	1,577		20	263
	21 February 2006			40	252,000	290	1,291		20	1,619
	14 June 2006			56	210,000	297	2,425		20	2,204

[a] Dates shown in bold are the times of enrollment sample collection. NA, not applicable; ND, not done.
[b] Reciprocal serum dilution that gives 50% neutralization.

the V1-to-V5 region inversely correlated with the autologous NAb titer. In addition, we also found an inverse correlation between the number of PNGS and the autologous neutralization titer. These data suggest that increased variable loop lengths and N-linked glycosylation may protect the vulnerable areas of the envelope from antibody recognition.

Despite the potency of the autologous NAb response, these antibodies had limited breadth even after 12 months of infection. This is similar to what was reported recently by Li et al. for subtype C infection, which is in contrast to the higher degree of breadth reported for subtype B-infected individuals (8). The extremely narrow specificity of the early NAb response in subtype C infection suggests that these antibodies may be targeted to variable regions, which would include V1-V2, V4, and V5. Interestingly, we found that the sensitivities of early subtype C envelopes to heterologous neutralization inversely correlated with the length of the V1-V2 region. This inverse correlation suggests that the epitope(s) involved in cross-reactivity may be occluded by this loop, consistent with multiple previous studies (12, 15, 18). This is in contrast to the correlation we observed with autologous responses where increased glycosylation in the V1-V2 region, but not V1-V2 length, was associated with less potent responses. Glycosylation may offer protection of the V1-V2 region from antibodies, and therefore increased numbers of PNGS may result in reduced autologous NAb titers. Taken together, these correlations suggest that the V1-V2 region occludes more-conserved epitopes involved in cross-reactivity, whereas the autologous response may be targeted more to type-specific areas, possibly V1-V2 itself.

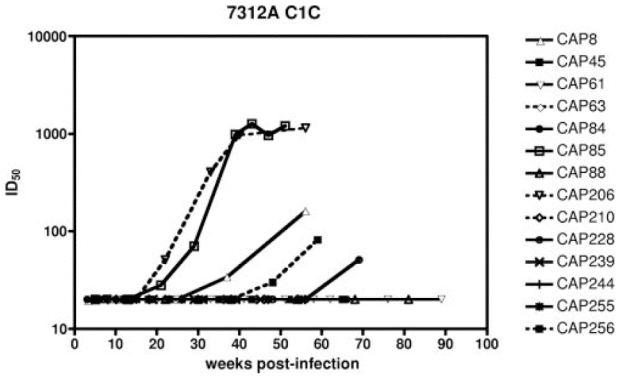

FIG. 8. MPER NAb responses in acute HIV-1 subtype C infection. Serum samples from 14 acutely infected individuals were tested against an HIV-2 envelope containing an HIV-1 MPER optimized for HIV-1 subtype C sequences (7312A CIC). Results are shown as ID_{50} values over time.

The envelope protein from patient CAP85 was the most sensitive to neutralization by both sera from within the CAPRISA cohort and other heterologous plasma samples and was excluded from the analysis shown in Fig. 6. The virus sensitivity profile was more akin to that of the primary viruses in the subtype C panel that had been cultured than to those of viruses cloned directly from plasma (9). Analysis of the gp160 sequence from the virus obtained from CAP85 demonstrated a number of changes at highly conserved positions, including A219T, M434V, and P437N. These mutations were present in all four functional clones obtained from this individual, which were all highly sensitive to neutralization (data not shown). The residue M434 is embedded in the bridging sheet of gp120 (7), and substitutions at this position have major effects on the sensitivity to CD4i antibodies (1, 22). This suggests that the M434V substitution in the CAP85 virus may expose the coreceptor binding site, making it especially sensitive to neutralization. This virus, however, did not have a general neutralization-sensitive phenotype, as it was not particularly sensitive to sCD4.

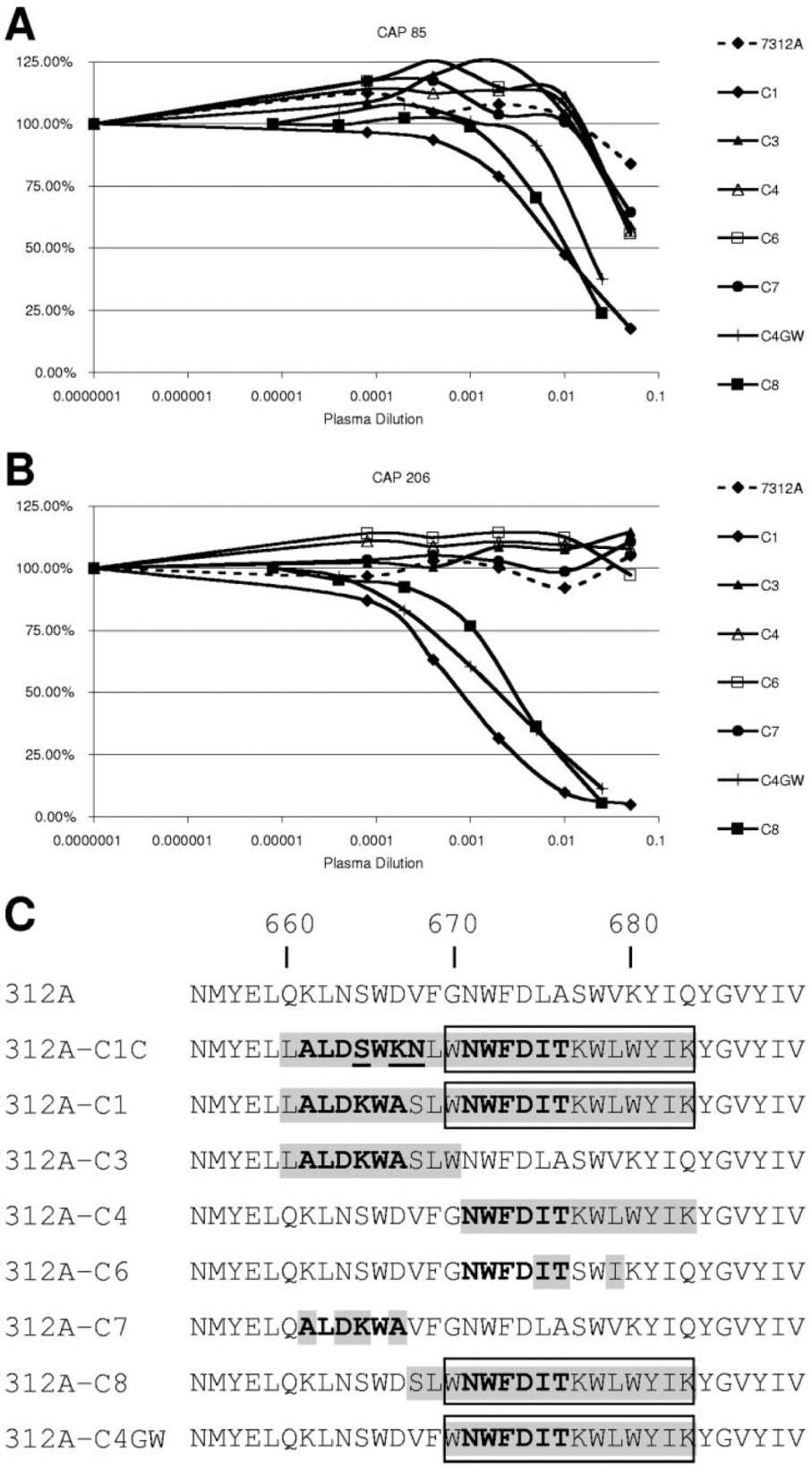

FIG. 9. Mapping of anti-MPER neutralizing activity in two patients. Serum samples from patients CAP85 (A) and CAP206 (B) were tested against eight HIV-2 chimeric viruses containing HIV-1 MPER fragments (C) plus the 7312A control virus. Results are shown as percentages of inhibition. The inserted fragments or mutated residues are shaded for each construct. The amino acids involved in the 2F5 and 4E10 epitopes are represented in bold. The common regions among the constructs where activity was found are boxed.

CD4i antibodies developed within a median of 12 weeks in most participants, often before the appearance of an autologous response. In three women, CD4i antibodies were detected very early, within 2 to 5 weeks of infection. Interestingly, all three women had low CD4 T-cell counts, and two were classified as having rapid disease progression, suggesting that these antibodies were not controlling viral replication. Surprisingly, these antibodies failed to neutralize their autologous envelopes in the presence of sCD4. It would be of interest to examine earlier envelope clones, if possible, to determine if they showed sensitivity to CD4i antibodies. If so, this could suggest that CD4i antibodies function to constrain the virus to remain CD4 dependent, as proposed previously (1). Overall, the contribution of CD4i antibodies to breadth remains unclear, as we found only a weak correlation between the magnitude of this response and the ability to neutralize heterologous viruses.

Antibodies to the MPER developed in a much smaller proportion of women than did CD4i antibodies. Only two women (CAP85 and CAP206) developed high titers, both within 6 months of infection. Epitope mapping demonstrated that these antibodies were not 4E10-like, despite their epitopes overlapping the 4E10 epitope. Both of these sera were unusual in their ability to neutralize the heterologous virus COT6, which has been shown to be highly sensitive to the gp41 MAb 4E10 (E. Gray, unpublished). Since sensitivity to 4E10 may be due to a more-exposed MPER, these data suggest that the neutralization of COT6 by CAP85 and CAP206 sera may be due to anti-MPER antibodies. However, other than COT6, these sera were not especially cross-reactive to heterologous viruses, suggesting that these anti-MPER antibodies do not generally confer breadth of activity, at least during the first year of infection.

In summary, the results reported here confirm previous data indicating that the autologous neutralizing response in HIV-1 subtype C-infected individuals develops to a high titer within months of infection but remains strain specific even after 1 year. The correlation between V1-V2 length and virus sensitivity to heterologous but not autologous neutralization suggests that strain-specific antibodies target areas distinct from those targeted by cross-neutralizing antibodies. The specificities of these early autologous antibody responses remain to be determined.

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CHAPTER SIX

SUMMARIZING DISCUSSION AND CONCLUSIONS

The induction of neutralizing antibodies constitutes a major focus for the development of an HIV-1 vaccine, as it remains the only immune response that has been proven capable of completely blocking virus entry in animal models. Despite the fact that HIV-1 has evolved many strategies to avoid antibody recognition of the conserved regions in the envelope glycoprotein, the existence of a few broadly neutralizing MAbs, such as IgG1b12, 2G12, 2F5 and 4E10, highlights the presence of "weak spots" that can be targeted for vaccine design. These nMAbs were isolated from subtype B infected patients and their epitopes have mainly been characterized in viruses from this subtype. Given that subtype C viruses are responsible for the majority of the HIV-1 infections worldwide, a growing number of researchers have expanded their interest to include these viruses. In this context, this work focuses on the study of the neutralizing antibody epitopes in the subtype C envelope glycoprotein.

During the course of this study several subtype C viruses were characterized for their sensitivity to neutralization by the above mentioned broadly nMAbs. An overview of the neutralization efficacy of these antibodies for viruses from our laboratory is shown in Figure 6.1. It encompasses 26 subtype C envelope clones, including the paediatric viruses described in Chapter Two, the 14 acute infection viruses studied in Chapter Five and five other viruses isolated from chronically infected individuals. In summary, 4E10 constituted the most broadly reactive neutralizing antibody, followed by IgG1b12 that was able to neutralize around fifty percent of the tested viruses. 2G12 and 2F5 neutralized subtype C viruses poorly, in agreement with similar studies by other groups (Binley *et al.*, 2004, Li *et al.*, 2006b). The paucity of broadly nMAbs against some HIV-1 clades calls for further efforts in search of new antibodies. Furthermore, these reagents might point to new potential targets for immunogen design. Given that the existing nMAbs revealed vulnerable structures on the envelope glycoprotein, it is appropriate to speculate that other

neutralizing antibodies that recognize similar epitopes, on non-subtype B envelopes, might also have broad activity, *i.e* antibodies that recognize a glycan arrangement or antibodies against the MPER. On the other hand, such antibodies may not be commonly found in HIV-1 infected individuals, as they have all shown extremely unusual features. These extraordinary structural adaptations (Burton *et al.*, 2005) may depend on host genetic elements, such as the germline antibody repertoire or the case of the flexible hinge region of 2G12. Others have suggested that the capacity of the nMAbs 2F5 and 4E10 to recognize the lipid bilayer, as a mechanism to access the MPER, is associated with the polyreactivity of these antibodies (Haynes *et al.*, 2005a). This suggests that B-cells carrying such autoreactive specificities normally would suffer clonal deletion, anergy or receptor editing (Haynes *et al.*, 2005b). If this is indeed the case, it constitutes a major challenge to the field and a call for a better understanding of the B-cell populations where such unusual antibodies originate, as it may inform us how to manipulate the immune system to induce such specificities. However, a recent study showed that anti-HIV nMAbs are not exceptionally polyreactive, challenging the notion that autoantigen mimicry constitutes a mechanism of immunoevasion by HIV-1 (Scherer *et al.*, 2007).

In our study, resistance to the 2F5 MAb was explained, in most cases, by substitutions in the K665 residue to serine (S). Interestingly, the motif DKW of the intact 2F5 epitope was present in four viruses that were not sensitive to neutralization by this nMAb (Appendix A). However, all three viruses had a glutamine (Q) residue at position 667, which may explain their resistance to 2F5. Data from previous studies concur with this reasoning, as viruses where position 667 was substituted to Gln or Asp were found to be resistant despite the presence of an intact DKW motif (Binley *et al.*, 2004, Li *et al.*, 2005, Li *et al.*, 2006b). These substitutions may affect the β-turn conformation recognized by 2F5, as this residue is involved in the formation of one of the three intra-molecular hydrogen bonds formed in

this epitope (Ofek *et al.*, 2007). While the alanine residue at position 667 is relatively conserved in subtype B strains, this position is more polymorphic amongst subtype C viruses with common substitutions to K, Q, or D.

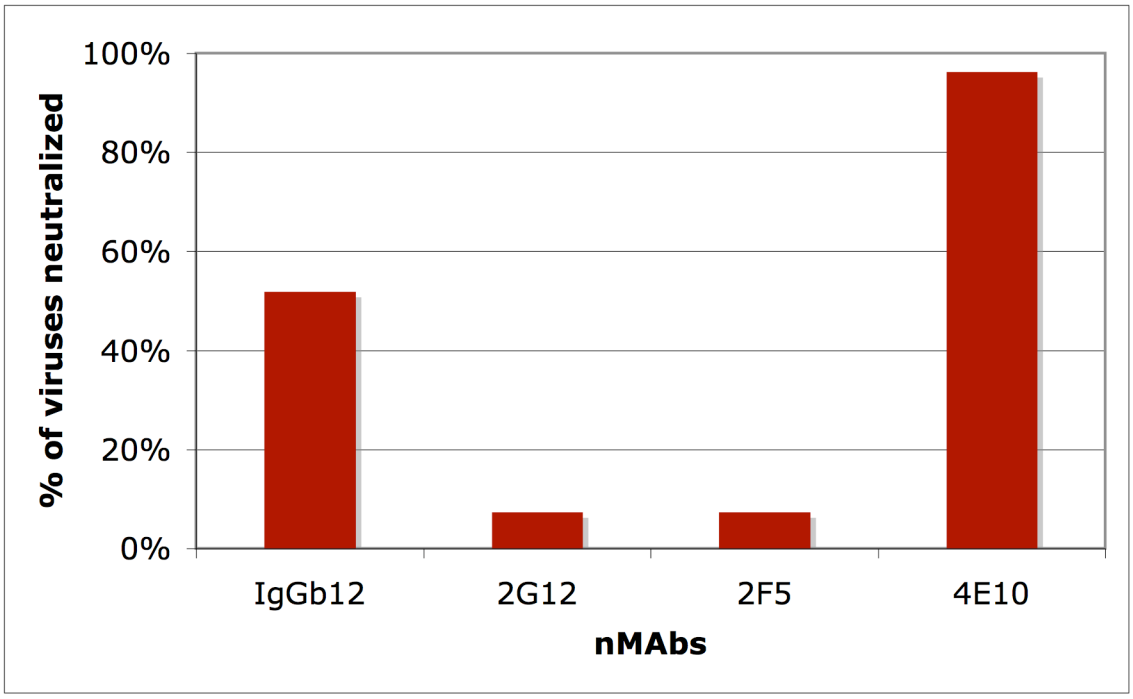

Figure 6.1: Percentage of viruses sensitive to neutralization by the broadly nMAbs IgG1b12, 2G12, 2F5 and 4E10. A total of 26 subtype C viruses from our laboratory were tested against the nMAbs. Neutralization titers and specific antibody epitope sequences for each of the viruses are detailed in Appendix A.

The common lack of reactivity of the nMAb 2G12 against subtype C viruses has been attributed to the absence of a glycosylation site at position 295, as reinforced in Figure 6.1 and Appendix A. The results presented in Chapter Three showed that the addition of a glycan at this position did not effectively reconstitute the 2G12 epitope in two subtype C viruses, even when other ancillary glycans were present, suggesting that this epitope may be displayed in a distinct conformation in the context of a subtype C core. Given that a subtype C gp120 structure has not been resolved, we speculate that it may differ from the well-characterized subtype B gp120 core. The following evidence supports this hypothesis; some positively selected sites found in subtype C envelopes are under negative selection in

subtype B viruses (Travers *et al.*, 2005), some of which map to the C3 region of gp120 (Choisy *et al.*, 2004, Gaschen *et al.*, 2002). This region includes the α2-helix, which has been reported to have a very conserved amphipathic nature in subtype C viruses in comparison to its more hydrophobic subtype B counterpart (Gnanakaran *et al.*, 2007). This suggests that this helix is more exposed in subtype C envelope glycoproteins. In contrast, the V3 loop of subtype C viruses is very conserved. A different pattern of glycosylation also distinguishes these subtypes (Zhang *et al.*, 2004), such as the differential occurrence of glycosylation sites at positions 295 and 442. Taken together, these observations argue that the envelope glycoproteins of these subtypes may have distinct antigenic properties. This could have resulted from different selection pressures throughout their epidemic history, such as the mode of transmission, host genetics and presence of concomitant communicable diseases in these populations. Accordingly, differential glycan arrangements might affect viral tropism or affinity for molecules involved in virus binding and transport, such as DC-SIGN, mannose binding protein or syndecans (Gallay, 2004, Nguyen and Hildreth, 2003, Sanders *et al.*, 2002a). In our study, the glycosylation mutants did not show apparent changes in viral entry; however, the effect on viral tropism or coreceptor affinity was not assessed. Furthermore, a recent study reported that deletion of a glycan increased macrophage tropism (Dunfee *et al.*, 2007), while another study showed that *in vitro* generated 2G12 resistant viruses, became more sensitive to neutralization by several mannose-specific lectins (Huskens *et al.*, 2007). Collectively, these observations support the necessity to explore whether the distinct glycosylation patterns and, in general, the differences between envelope glycoproteins of various subtypes could be the result of adaptations to different environmental conditions.

All but one of the viruses tested here were sensitive to 4E10, supporting the cumulative evidence that this is the broadest neutralizing antibody available. The one exception was

clone TM20.13, which was further characterized in the work described in Chapter Four. This study reinforced the importance of the MPER as a vaccine target, as it suggests that neutralizing antibodies to this region can indeed exert pressure on the virus, as evidenced by the appearance of escape variants. Due to the small scope of this study, with only one blood sample from this interesting case, it is not clear how escape from such antibodies occurred and how it affected disease progression. We explored the incidence of anti-MPER antibodies in an HIV-1 subtype C acute infection cohort. As presented in Chapter Five, four of the 14 studied individuals developed anti-MPER antibodies, however, their sera were not able to neutralize multiple heterologous viruses, suggesting that these antibodies were not conferring neutralization breadth. On the other hand, the role of these anti-MPER antibodies in autologous neutralization is unknown, and it will require further study. However, given the conservation of the MPER region, it is unlikely that these antibodies mediate type-specific recognition.

The CAPRISA acute HIV-1 infection cohort gave us the opportunity to scrutinize the role of the neutralizing antibody response in natural infection. The work presented in Chapter Five is the beginning of a profound study of the evolution of neutralizing antibody responses in subtype C infection. In here, neutralization of the early autologous virus was detected only after 19 weeks post-infection, despite anti-HIV antibodies being detected as early as two weeks, anti-gp41 antibodies at 5-10 weeks and anti-V3 antibodies at 3-10 weeks post-infection (Alam *et al.*, 2007, Moore *et al.*, 2007). Furthermore, these autologous neutralization responses were highly type-specific with limited cross-reactivity even at two years post-infection (unpublished observations). The study of the specificities associated with this narrow neutralization response has been the subject of a recent study, which suggests that the C3-V4 region is a major target of type-specific autologous neutralizing antibodies (Moore *et al.*, 2007). It is not clear if this finding can be extended

to other HIV-1 subtypes, therefore, it is important to perform a similar study at least in subtype B infected individuals.

Interestingly, in three patients, CD4i antibodies were detected very early in infection. Although the role of these antibodies is not clear, it has been proposed that CD4i antibodies may influence viral pathogenesis by constraining the virus to CD4 dependence. A comprehensive analysis of envelope sequences amplified from single genomes suggests that during acute infection there is no immune pressure on the virus as *env* diversity follows a Poisson distribution (Keele *et al.*, 2007). Given that the inferred transmitted viruses showed CD4 dependence, no evidence is available to suggest that transmission of early virus replication favours CD4 independent variants.

It is a conundrum for the field as to why there is a delay in the production of neutralizing antibodies to HIV-1 and particularly broadly cross-reactive antibodies. While in general the induction of neutralizing antibodies requires time to achieve affinity maturation, in early HIV-1 infection this period is prolonged because of the absence of an efficient T-helper response and a general non-specific immunoactivation (Brenchley *et al.*, 2006). Furthermore, B-cells producing broadly cross-reactive neutralizing antibodies may have to compete with non-neutralizing antibodies to immunodominant epitopes and perhaps with non-neutralizing antibodies to the same epitope (Alam *et al.*, 2007) or even type-specific neutralizing antibodies. While the initial vaccine response has to deal only with the latter, the rapid activation of memory B-cells upon infection is another stumbling block in HIV-vaccine development. Therefore, extensive research is needed into the understanding of the B-cell regulatory pathways that determine their activation, differentiation and establishment of long-lived plasma cells as well as memory B-cells.

The continued monitoring of individuals enrolled in the CAPRISA study will allow us to the study the specificities of antibodies conferring cross-reactivity as well as provide useful

samples from which new nMAbs could be obtained. Further studies will address the question of what constitutes neutralization breadth. While some studies suggest that neutralization breadth in HIV-1 infected patients is due to the presence of very few antibody specificities targeting vulnerable areas (Dhillon *et al.*, 2007, Li *et al.*, 2007) another possibility is that the accumulation of multiple "type-specific" antibodies confers breadth. This is an important question to address as if the latter is found to be the case, then the induction of a broad neutralizing antibody response will constitute even more of a challenge that is currently appreciated. Furthermore, such data may inform additional strategies to be followed in the design of an HIV vaccine.

APPENDIX A Review table of subtype C viruses neutralization by nMAb

Neutralization titers and amino acid sequences of the epitopes of broadly nMAb in cloned subtype C envelope genes

ENV clone	IgG1b12	2G12*					2F5**					4E10**										
	IC50	IC50	295	332	392	339	386	IC50	662	663	664	665	666	667	668	IC50	671	672	673	674	675	676	677	678	679	680
			Nx(S/T)	Nx(S/T)	Nx(S/T)	Nx(S/T)	Nx(S/T)		E	L	D	K	W	A	S		N	W	F	D	I	T	N	W	L	W
RP1.12	50.0	50.0	VCI	N/S	NGT	NKT	NTS	50.0	A	.	.	R	.	N	N	13.2	S	.	.	S	.	.	.	.	.	.
RP6.6	0.9	50.0	ECT	NIS[&]	NNS	NTT	NTT	50.0	A	.	.	N	.	N	S	17.1	.	.	.	N	.	.	.	.	.	.
RP4.3	11.9	50.0	VCT	N/S	NRT	NNT	DTS	50.0	A	.	.	S	.	N	N	45.8	.	.	.	S	.	.	K	.	.	.
COT9.6	50.0	50.0	VCT	N/S	NTS	NRT	NTS	50.0	A	.	.	S	.	K	N	3.0	S	.	.	.	.	.	K	.	.	.
COT6.15	3.4	50.0	VCT	N/S	NGT	NKT	NTS	50.0	A	.	N	S	.	Q	N	35.9	S	.	.	S	.	.	.	.	.	.
TM7.9	0.2	50.0	VCT	N/S	NRR	NKT	NTS	50.0	A	.	.	S	.	K	N	34.5	.	.	.	S	.	S	.	.	.	.
TM3.8	50.0	50.0	MCT	N/S	NST	NKT	NTS	50.0	A	.	.	S	.	K	N	21.6	S	.	.	N	.	S	.	.	.	.
TM20.13	13.4	50.0	VCT	N/S	NTS	NKT	NSI	30.9	A	.	.	.	.	N	N	50.0	S	.	L	S	.	S	.	.	.	.
SW7.14	50.0	50.0	VCT	N/S	NTS	NKT	NRT	50.0	A	.	.	S	.	N	.	2.1	S	.	.	.	.	.	S	.	.	.
Du174.15	0.4	50.0	VCT	N/S	NTS	NKT	NSM	50.0	A	.	N	.	.	Q	N	9.0	G	.	.	S	.	.	.	.	.	.
Du179	18.3	50.0	VCT	N/S	NTT	NGT	NSS[&]	50.0	A	.	.	.	.	Q	N	5.7	S	.	.	S	.	.	.	.	.	.
525-2-8	50.0	50.0	VCT	N/S	NTS	LTT	NSA	50.0	A	.	.	S	.	N	N	7.1	.	.	.	G	.	.	K	.	.	.
536-10-7	50.0	50.0	MCT	N/S	NTS	NDT	NNT	50.0	A	.	.	S	.	Q	N	4.4	S	.	.	S	.	.	.	.	.	.
CAP8 6F	13.7	50.0	TCT	N/S	NTS	TET	NES	50.0	A	.	.	S	.	K	N	7.1	.	.	.	.	.	.	.	.	.	.
CAP45 G3	0.5	50.0	VCR	N/N	NTT	NRT	KWS	50.0	A	.	.	S	.	N	N	0.7	.	.	.	N	.	.	.	.	.	.
CAP61 F10	50.0	50.0	ECV	N/S	NTS	NKT	NGT	50.0	A	.	.	.	.	Q	N	18.8	S	.	.	.	.	.	.	.	.	.
CAP63 A9	24.7	50.0	VCA	N/S	NTS	NTT	NST	50.0	A	.	.	.	.	Q	N	2.8	S	.	.	N	.	S	H	.	.	.
CAP84 32	50.0	50.0	VCT	N/S	NTT	EKT	NLN	50.0	A	.	.	S	.	N	.	1.1	.	.	.	S	.	.	K	.	.	.
CAP85 9	14.4	50.0	VCT	N/S	NTS	NNT	NST	0.3	A	.	.	.	.	.	N	0.5	.	.	.	.	.	.	.	.	.	.
CAP88 B5	19.4	50.0	VCI	N/T	NTS	IAT	NEN	50.0	A	.	.	S	.	N	N	0.6	.	.	.	N	.	.	Q	.	.	.
CAP206 8	50.0	50.0	VCT	N/L	NTT	NTT	NEN	50.0	A	.	.	S	.	K	N	2.3	.	.	.	.	.	.	K	.	.	.
CAP210 E8	7.8	50.0	TCI	N/S	NTT	NET	NST	50.0	A	.	.	.	.	Q	.	1.0	S	.	.	S	.	S	S	.	.	.
CAP228 51	9.9	50.0	VCT	N/S	NTT	NKT	NEN	50.0	A	.	.	S	.	N	N	9.3	T	.	.	N	.	S	.	.	.	.
CAP239 G3	50.0	4.6	NCT	N/S	NTS	NET	NGT	50.0	A	.	.	S	.	N	.	9.2	.	.	.	S	.	.	.	.	.	.
CAP244 D3	50.0	50.0	VCT	N/D	NTS	NKT	NNT	50.0	A	.	N	S	.	D	.	0.8	S	.	.	S	.	.	K	.	.	.
CAP255 16	50.0	50.0	NCI	N/S	NTS	DKT	NST	50.0	A	.	.	S	.	N	N	1.0	T	.	.	.	.	.	.	.	.	.
CAP256 7C	2.4	10.8	NCT	N/S	NTS	EKT	NGT	50.0	A	.	.	S	.	N	.	5.8	.	.	.	S	.	S	T	.	.	.

*Predicted N-linked glycosylation sites are bolded and italicised
**Residues crucial for 2F5 and 4E10 MAb activity are bolded and italicised
[&]The PNG site is moved one aa downstream
2F5 epitopes with intact DWK motif have been showed

APPENDIX B Ethical Clearence

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Gray

CLEARANCE CERTIFICATE **PROTOCOL NUMBER MO60637**

PROJECT Characterisation of neutralizing antibody epitopes on HIV-1 envelope glycoproteins to support vaccine design

INVESTIGATORS Mrs ES Gray

DEPARTMENT AIDS Research Unit

DATE CONSIDERED 06.06.30

DECISION OF THE COMMITTEE* APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.08.16 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr L Morris

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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